

SYMBOLAE BOTANICAE UPSALIENSES IX:2
ARBETEN FRÅN BOTANISKA INSTITUTIONERNA I UPPSALA
UTGIVNA AV PROF. E. MELIN OCH PROF. J. A. NANNFELDT

STUDIES IN THE
CELLULOSE DECOMPOSITION
BY CYTOPHAGA

BY

GÖSTA FÅHRÆUS



UPPSALA
A.-B. LUNDEQUISTSKA BOKHANDELN
(I DISTRIBUTION)

SYMBOLAE BOTANICAE UPSALIENSES

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STUDIES IN THE
CELLULOSE DECOMPOSITION
BY CYTOPHAGA

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Preface.

When the experimental work underlying the present paper was first begun in the autumn of 1937, its scope was the study of the bacterial degradation of cellulose from an enzymatic point of view. My interest was directed to this subject by Professor CHR. BARTHEL¹, and an outline for the investigation was suggested by him and by Professor R. NILSSON.² As a suitable material the genus *Cytophaga* was chosen, since it was apparently very wide-spread in nature and of great importance for the decomposition of cellulose in soil.

For a successful prosecution of the enzymatic studies it was necessary to attempt a culturing of the bacteria on a scale large enough to allow the preparation of sufficient amounts of cell substance. During the preliminary literature studies and experiments it became evident, however, that the general physiology of the bacteria concerned was in fact very incompletely known and that the culturing on a larger scale did not seem promising under these circumstances.

On these grounds it seemed necessary to me, as an introduction to the enzymatic studies planned, to make some investigations into the general physiology of the bacteria in question with the intention of ascertaining the optimal conditions for growth. In the course of this work my interest was attracted by some particular features in the physiology of *Cytophaga*, and a number of experiments were carried out in order to elucidate the very confusing details regarding the utilization of soluble carbohydrates by *Cytophaga*. Concurrently my attempts to obtain dry preparations of bacteria in sufficient amounts were carried on, and it proved possible to reach certain conclusions as to the course of cellulose decomposition through experiments with such preparations. Since the methods of culturing *Cytophaga* and producing dry bacteria had been improved, a number of enzyme experiments could finally be made.

Concurrently with the present work investigations dealing in part with the same problems have been carried out in other countries. Among

¹ Late Director of the Institute of Microbiology of the Agricultural College of Sweden.

² Present Director of the Institute of Microbiology.

these may be mentioned those of WALKER and WARREN (1938), NORMAN and BARTHOLOMEW (1940), STANIER (1942), FULLER and NORMAN (1943) and HARMSSEN (1946). Owing to the war, several of the papers in question did not become available to me until many years after their publication, unfortunately. In consequence, the results obtained by these authors could not always be considered in the experiments of the present writer. The results arrived at by these authors seem to agree with my own in many respects, however.

The bacterial group treated in this paper presents much of interest in various respects. The morphology is especially remarkable, and this has led to extreme confusion regarding the systematic position of the whole group. Much work has been done during recent years to clear up these problems. Since the present author has studied the morphological questions only very superficially, this aspect will be touched upon only inasmuch as it appears valuable for an understanding of the physiological problems. For further information the reader is referred to recent surveys, for instance those of STANIER (1940, 1942 b) and NORMAN and FULLER (1942).

*

The present investigation was carried out partly at the Institute of Physiological Botany of the University, Uppsala, and partly at the Institute of Microbiology of the Agricultural College of Sweden, Uppsala — Ultuna.

It is my pleasant duty to proffer my warmest thanks to Professor ELIAS MELIN of the University of Uppsala for the lively interest he has shown in my work, for valuable discussions, and for the laboratory facilities and kind support he has given me in every respect. I also wish to thank him for including this thesis in "*Symbolae Botanicae Upsalienses*".

To my other greatly esteemed professors in Botany, in Stockholm and Uppsala, Professor O. ROSENBERG, Professor M. G. STÅLFELT and Professor J. A. NANNFELDT, I also desire to express my deep gratitude for valuable instruction and good advice during my university years.

A very considerable part of the investigation has been carried out at the Institute of Microbiology of the Agricultural College. To the late and present Directors of this Institute, Professor CHR. BARTHEL and Professor R. NILSSON I am very much indebted for placing laboratory accommodation and assistants at my disposal. I greatly appreciate the encouragement and support that Professor NILSSON has given me during my six years in his Institute.

For valuable discussions, advice and criticism I cordially thank my friend Mr. F. ALM, Fil. Mag.

To Miss M. EK, Miss K. LINDBLAD, Fil. Mag., and especially Mr. H. BLOM, who have been my assistants at different periods in the course of this research, I express my warmest thanks. My sincere thanks are also due to Mrs. E. BOLIN for her skilful drawing of the figures in this thesis.

The revision from a linguistic point of view has been made by Miss K. M. PAIN, B. A., Fil. Kand. For the great care she has bestowed on this work I wish to convey my best thanks.

Finally, I take this opportunity to thank Dr. L. FRYKHOLM and Miss A. GUILLETMOT, Fil. Kand., of the Library of the Agricultural College, who have greatly facilitated my work.

My investigations have been carried out with the aid of grants from the "Gustaf och Anna Retzius' fond för vetenskaplig forskning" and the "Hahnska fonden" of the Royal Swedish Academy of Sciences and from "Statens Tekniska Forskningsråd", for which I wish to express my sincere gratitude.

I. Taxonomy and Morphology of *Cytophaga*.

Survey of the literature.

The cellulose-decomposing microorganisms have been conventionally divided into three main types: 1) the anaerobic, 2) the thermophilic, and 3) the aerobic.

Our present knowledge of the microorganisms concerned allows a more differentiated division on morphological grounds, and it must be admitted that especially the last of the above-mentioned groups is rather heterogeneous. The groups also overlap, since there exist a number of facultative anaerobes. The thermophilic group, in addition, seems to comprise both anaerobic and aerobic forms. However, the division has certain arguments in favour of it, as the methods used in studies of the three groups differ in essential respects. The investigators have in general confined themselves to one group, and the division thus has a historical justification. It does not, however, imply that the course of decomposition is chemically different in the three types.

The first important investigations, where the decomposition of pure fibre cellulose was in fact established, were made with anaerobic bacteria in the laboratory of Hoppe-Seyler by POPOFF (1875) and others. Their work was then continued by OMELIANSKI (1895), who created an important basis for all later investigations into the anaerobic cellulose decomposition. Regarding the bacteria belonging to this group, which has not been studied by the present author, the reader is referred to reviews by SIMOLA (1931 a) and POCHON (1943). All species so far described seem to be spore-forming rods, which should consequently be referred to the genus *Clostridium*.

The aerobic cellulose-decomposing microorganisms represent a far more heterogeneous group. It comprises such widely different organisms as fungi, actinomycetes and bacteria. Within each of these groups there are again a great number of cellulose-decomposing types. In the present work the fungi and actinomycetes will on the whole be left out of consideration.

The first discovery of aerobic, mesophilic cellulose-decomposing bacteria was made by VAN ITERSON (1903), who was apparently also

the first to show that numerous moulds are capable of attacking true fibre cellulose. Since the days of VAN ITERSON a great variety of bacteria have been described as being able to decompose cellulose aerobically, e. g., spore-forming rods (SIMOLA 1931 a), non-spore-forming rods (KELLERMAN and collaborators 1912, 1913), vibrios (WINOGRADSKY 1929), Corynebacteria (JENSEN 1940), true Myxobacteria (H. and S. KRZEMIENIEWSKI 1937) and finally the cytophagas (HUTCHINSON and CLAYTON 1919). Regarding these groups compare the review by JENSEN (1940).

Most attention has been paid to the cytophagas during recent decades. This is not only due to the fact that they appear to play an important rôle in the soil, where they are extremely common, but also to their very interesting and often enigmatic morphological and physiological features.

The above-mentioned work by VAN ITERSON, and in addition those of MERKER (1912) and MÜTTERLEIN (1913), probably dealt with *Cytophaga* species, but the lack of pure cultures and the incompleteness of the descriptions given make this a matter of some doubt.

The fundamental work on the cytophagas, by the Englishmen HUTCHINSON and CLAYTON, appeared in 1919. In this publication a detailed and complete study of the morphological and physiological features of the bacteria in question was given for the first time. The newly isolated strain was named "*Spirochaeta cytophaga*", as it possessed morphologically "greater affinities with the *Spirochaetoideae* than with the bacteria".

It was found that the predominating forms in young cultures were simple sinuous filaments, $3 \times 0.3 - 0.4 \mu$. These cells were transformed into thicker and less filamentous forms with increasing age, this process resulting in the formation of spherical cells, "sporoids". Finally the sporoid gave rise to the thread form again. Some of the remarkable physiological features of *S. cytophaga* are outlined in the following words: "*S. cytophaga* appears to be specific in its relation to carbon sources and its requirements are only met by cellulose. Not only are other compounds — higher alcohols, organic acids and various carbohydrates — unsuitable for nutrition of the organism, but some, namely those compounds possessing marked reducing properties, have been found to be toxic even in very low concentrations . . .".

The morphological findings of HUTCHINSON and CLAYTON provoked much controversy in the years that followed. WINOGRADSKY (1929, 1932) criticized the development cycle indicated and explained the "sporoid" as a mere contaminant. On the other hand, KRZEMIENIEWSKA (1930, 1933), STAPP and BORTELS (1934) and others, confirmed the main view taken by the English authors. KRZEMIENIEWSKA (1933) gave the final solution of the problem in establishing the occurrence of two types of *S.*

cytophaga, some strains possessing microcysts (the "sporoid" of HUTCHINSON and CLAYTON), and some strains being without microcysts. Later investigations into the morphology of *S. cytophaga* have confirmed KRZEMIENIEWSKA's statements entirely (STAPP and BORTELS 1934, IMŠENECKI and SOLNCEVA 1936, JENSEN 1940, STANIER 1940, 1942 b, FULLER and NORMAN 1943 a). For a more complete review of the facts in this connection the reader is referred to these publications, especially those of STANIER. An extensive review of the literature has been given recently by HARMSEN (1946), although in Dutch.

The generic name *Cytophaga* was first adopted by WINOGRADSKY (1927), since it was clear that the classification among the *Spirochaetales* was based only upon a superficial similarity to these organisms. WINOGRADSKY expressed no definite opinion as to the taxonomic position of *Cytophaga*. KRZEMIENIEWSKA (1930) first pointed out the near relationship to the Myxobacteria.

The position in the neighbourhood of this group is now generally accepted. Apart from the formation of microcysts in certain species, the high flexibility and the creeping movement of the vegetative cells, pointed out already by HUTCHINSON and CLAYTON, are very characteristic properties in *Cytophaga* as also in the real Myxobacteria. Most Eubacteria, on the other hand, have a rigid cell wall.

STANIER (1940, 1942 b) proposes the creation of a new family, *Cytophagaceae*, within the order *Myxobacteriales*, where all amicrocystogenous species should be placed. To these belong the type species *Cytophaga Hutchinsoni* (WINOGRADSKY 1929) and some additional strains described by WINOGRADSKY, STAPP and BORTELS (1934), WALKER and WARREN (1938), JENSEN (1940), and others. The microcyst-forming species are placed in a new genus *Sporocytophaga* within the family *Myxococcaceae*. The type species is *Sp. myxococcoides* (KRZEMIENIEWSKA 1933, emend. STANIER). Identical or nearly related are the original *Spirochaeta cytophaga* (HUTCHINSON and CLAYTON 1919), further *C. globulosa* (STAPP and BORTELS 1934) and *C. Hutchinsonii* (IMŠENECKI and SOLNCEVA 1936). Strains described by DUBOS (1928) and JENSEN (1940) also belong here.

It seems to the present author a little doubtful whether this division into separate families on the basis of the occurrence of microcysts is fully justified, since it is not impossible that this property can be lost (STANIER 1942 b p. 153). IMŠENECKI and SOLNCEVA (1945) adopt the genera *Cytophaga* and *Sporocytophaga* but suggest the classification of the two genera together in a new family: *Promyxobacteriaceae*.

STANIER (1940, 1941) first discarded the idea of obligate cellulose decomposition in *Cytophaga* by inclusion on morphological grounds of two

newly discovered marine species; these attack not only cellulose but also agar and some simple sugars. Some chitin-destroying bacteria were also included in the genus. FULLER and NORMAN (1943 a) have described some strains which are only slightly capable of decomposing fibre cellulose but which attack cellulose dextrans and simpler carbohydrates of different kinds. One versatile strain formed microcysts and was placed in *Sporocytophaga* by the same authors. GARNJOBST (1945) has recently described a *Cytophaga* pathogenic to fish.

The morphological and taxonomical details briefly reviewed here may suffice for our purpose. When discussing the physiological problems in *Cytophaga* some earlier results with the strains mentioned above will be related. For further information about the morphology and taxonomy of the cytophagas, the reader is referred to the exhaustive reviews by STANIER (1940, 1942 b).

While the interpretation of the morphology of *Cytophaga* given by HUTCHINSON and CLAYTON was first sharply criticized but later proved true in most essential respects, their conception of the physiological features was first strongly supported by all investigators. Not until recent years have new experimental results appeared which have brought about a change in the view of the carbon requirements of *Cytophaga*. These recent investigations will be related in more detail on pp. 36 et sqq.

II. Material and Methods.

A. Material.

1. Bacterial strains used.

In the present investigation two *Cytophaga* strains have been mainly employed: *Cytophaga 3* (FÅHRÆUS 1941) and *Cytophaga globulosa* (STAPP and BORTELS 1934). Both these strains are microcyst-forming and conform on the whole with the type species (*Sporo*-)*cytophaga myxococcoides* (see p. 11 above and Pl. I—II). Differences have been observed in respect to pigment, sensitivity towards soluble carbohydrates, etc.

Cytophaga 3 was isolated in the autumn of 1937 from manured garden soil in the neighbourhood of Stockholm. With this strain the first experiments were performed. Morphologically it has not been studied by me in greater detail. The pigment is egg-yellow and assumes an orange-yellow tint when dissolved in alkali. The odour of the cultures is faint, somewhat acidic. This strain is less sensitive to glucose and other soluble carbohydrates than most strains earlier described. (See also p. 37.)

Cytophaga globulosa was obtained from Professor STAPP in 1939. Originally it was isolated from beech-wood litter in the vicinity of Berlin. The morphology and physiology of this strain were well known in several respects through the work of STAPP and BORTELS (1934). The pigment of this strain is greyish-yellow and not egg-yellow as in *Cytophaga 3*. When dissolved in alkali it becomes reddish. The odour is rather aromatic and somewhat resembles that of yeast. The composition of the "mucilage", the slimy substance formed extensively in *Cytophaga* cultures, seems to diverge from the normal type; this is further dealt with on p. 81.

During 10 and 8 years' cultivation respectively, the strains mentioned have not changed in any morphological or physiological respects. The environmental factors being unchanged, the quantitative decomposition of cellulose brought about also has a rate which is surprisingly similar on different occasions. I will refer to the experiments with *Cytophaga 3* and *C. globulosa* on pp. 48 and 66, the experiments in question being carried out with an interval of $3\frac{1}{2}$ years.

2. Isolation of *Cytophaga* 3.

Since this strain has been used in most earlier experiments, a brief report is given here of the isolation method used.

The first enrichment was effected by means of the method of HUTCHINSON and CLAYTON (1919). The procedure is simple: a filter paper partly immersed in a liquid mineral medium in a flask is inoculated with soil particles. At the first visible growth (yellow zones appearing around the particles) fibres were transferred to new flasks or tubes containing the same medium with filter paper strips. By repeated subculturing and by successive dilution cultures were finally obtained which were fairly pure.

For the final purification some of the plating methods recommended in the literature were tried, e. g. the silica gel plates of WINOGRADSKY (1929), the sand plates of KRZEMIENIEWSKA (1933), and the plates with clay pieces of RIPPEL and FLEHMIG (1933). Agar plates containing finely divided cellulose or filter paper strips placed on the surface were also tried. In most cases the nutrient medium used was that indicated by WAKSMAN and CAREY (1926), see below p. 21.

By using these methods — the best was in the opinion of the present author that of KRZEMIENIEWSKA — a culture was obtained which apparently contained only one additional bacterial rod besides *Cytophaga*.

Through investigations by JENSEN (1940) and STANIER (1942 b) published later, the drawback of substrates of the above-mentioned type has been clearly established. Two circumstances must be considered in the purification of the *Cytophaga* forms. First the cellulose must be very finely divided, for instance the cellulose precipitated according to SCALES' (1916) method. Secondly the medium (the agar, etc.) must be firm enough to inhibit contamination but at the same time weak enough to allow a creeping movement of the bacteria to take place.

The present writer would like to add another important reason for using a weak agar: as clearly shown in my own experiments, agar of 1.5 % concentration has a very inhibiting effect on the growth of several cytophagas. The inhibitory substance can be extracted by means of cold water, although much time is required for a thorough purification of the agar (see p. 70).

By using a 1 per cent agar with precipitated cellulose JENSEN (1940) and STANIER (1942 b) have succeeded in obtaining pure cultures of *Cytophaga* strains without great difficulties. When my purification attempts were made in 1937, these matters were not yet clearly understood. In my experiments the final purification was accomplished by using the heating method, also employed by DUBOS (1928), IMŠENECKI and SOLNCEVA (1936) and POLITI (1941). Heating for ten minutes at 56° C proved sufficient for

complete purification. The contaminating rod was killed readily at this temperature, while *Cytophaga* survived. By subculturing from the heated tube a pure *Cytophaga* culture was obtained.

The strain purified in this way utilizes cellulose, cellobiose, glucose and mannose as carbon sources (cf. p. 38). Growth has not been observed on any ordinary substrate (broth, malt extract agar, etc.), nor under aerobic or under anaerobic conditions. The microscopic picture agrees with that of a typical *Cytophaga*. The strain has shown very constant behaviour during ten years' culture. After subculturing on glucose for more than ten generations it has developed immediately at reinoculation on cellulose and has there exhibited entirely normal features. For these reasons the purity of the culture obtained seems to be ascertained. The same applies to *Cytophaga globulosa*, which has been cultivated by me for eight years. Any infections of the stock cultures, which may have occurred occasionally, have been readily eliminated by dilution in tubes.

Micromanipulation to obtain a one-cell culture of *Cytophaga* 3 was tried but was unsuccessful, as the some 50 or so single cells taken out failed to develop in the cellulose medium used.¹ At this stage of the investigation the culture conditions were perhaps not clear enough for a favourable result to be expected.

B. Methods of Cultivation.

1. General considerations.

In studies of microbial cellulose-decomposition a culturing method should be employed which makes it possible in a simple way to determine the cellulose consumed in a number of parallel samples. Scarcely any such quantitative studies in cultures of cellulose-decomposing bacteria had been carried out at the time when this investigation was commenced. The physiological experiments previously undertaken were mainly qualitative in nature. Thus in most cases (HUTCHINSON and CLAYTON 1919, STAPP and BORTELS 1934, etc.) the development in tubes containing nutrient medium with a strip of filter paper was studied, the growth and cellulose decomposition being estimated in a purely qualitative manner. For quantitative studies this method is not very suitable, although used for instance by KALNIŅŠ (1930) in studying *Vibrio* species and by BRADLEY and RETTGER (1927) in examining some *Cellulomonas* species. For current

¹ STAPP and BORTELS (1934) were likewise unsuccessful in their efforts to get a one-cell culture of *Cytophaga globulosa*.

transference of stock cultures and for many purposes, when no quantitative figures are required, this method is simple and appropriate.

In studying the metabolism of the highly aerobic organisms here concerned, it is necessary in some way or other to provide a sufficient supply of air. It has been pointed out repeatedly that the characteristic growth in tubes with paper strips clearly shows the strict aerobiosis in *Cytophaga*; the cellulose is attacked only in the air-water level, where the supply of both is best. Already some mms under this level no growth at all can be observed. Above this level, on the other hand, the moisture is unsatisfactory for growth. In order to secure a good supply of air some investigators (WINOGRADSKY 1929, H. and S. KRZEMIENIEWSKI 1937) have tried silica gel plates with cellulose placed on the surface in quantitative experiments with cellulose-decomposing bacteria. It is obvious that this method involves very great difficulties in methodical respects.

Cultivation in submerged culture has apparently never been tried. IMŠENECKI and SOLNCEVA (1936) stated that *Cytophaga* is capable of decomposing cellulose with a limited access of oxygen, but this has not been confirmed by others (JENSEN 1940, STANIER 1942 b). Thus the opinion seems to have arisen that it is impossible to cultivate these forms in submerged culture. This conclusion, however, appears to be premature. It may be true regarding the conditions in tubes, where the dissolved oxygen is soon exhausted and the rate of diffusion is very reduced (the first attack on the paper, however, always appears as minute yellow points *under* the water surface). But in flasks with a large liquid surface and with cellulose evenly dispersed in the liquid a considerable decomposition of the cellulose takes place, even without special aeration arrangements. This applies not only to *Cytophaga* but also to the equally strictly aerobic *Cellvibrio* species.

It is quite natural that an aeration increases the rate of the metabolic processes very markedly. WALKER and WARREN (1938) used, for instance, Kluver flasks for culturing *Cytophaga*. In their experiments oxygen was supplied from a gas cylinder and a very extensive decomposition was brought about in a short time. They give the figure 50 % decomposition in 7—8 days (29° C). The medium was in that case originally 0.5 % in respect to cellulose. In my own experiments I have achieved a decomposition of up to 80 % of the cellulose originally present in only 5 days, when using Kluver flasks with a 0.5 % suspension of cotton-wool in nutrient medium (30° C). Compare Tab. 16.

The statement of WALKER and WARREN that pure oxygen is necessary to obtain a rapid growth is quite erroneous. They have not tried an adequate aeration with air. In fact, this is enough to ensure a vigorous development in a short time. A supply of pure oxygen may stimulate the

growth further. Apparently a certain adaptation to higher oxygen pressures must first take place; in my own experiments with *Cytophaga 3* the growth was decidedly better at normal oxygen pressure, but once commenced the growth was favoured by a pure oxygen current. This is also known in the case of other bacteria, cf. RAHN and RICHARDSON (1942) for a discussion.

NORMAN and BARTHOLOMEW (1940) have cultivated *Cytophaga* and other cellulose-decomposing bacteria in aerated vessels and STANIER (1942 b p. 163) has achieved a very rapid cellulose consumption through shaking flasks with precipitated cellulose in a shallow liquid layer.

The present author made at an early stage of the investigation quantitative experiments with aerated flasks believing this to be a *sine qua non* when culturing the aerobic *Cytophaga* species. Such a procedure does not lend itself well to experiments with a great many flasks, however. The aeration will vary considerably in the different flasks, resulting in a less good agreement of the values obtained. In resting flasks without aeration it is, on the other hand, possible to get good parallel values, if the inoculation is made with a fairly great inoculum (see below). At optimal temperature the rate of cellulose decomposition is also quite sufficient to allow a measurable decomposition to take place within a few days.

2. Procedure followed.

In accordance with the above considerations usual Erlenmeyer flasks with a shallow layer of medium containing cellulose have been used for the culturing of *Cytophaga* in most quantitative experiments. As a rule 300 ml flasks of Jena glass containing 100 ml of medium (cf. p. 21) with 0.5 per cent cellulose have been employed. Under such conditions the cellulose decomposed after 30 days at 30° C has amounted to more than 50 per cent of the cellulose originally present.

The optimal temperature for the *Cytophaga* strains studied lies at c. 32—37° C. This applies both to *Cytophaga 3* (own results) and *C. globulosa* (STAPP and BORTELS 1934, p. 49). Owing to a limited supply of incubators at my disposal, the optimal temperature could not always be chosen in the experiments. In the first glucose experiments, for instance, only 25° C was used. Later generally 30° C has been used for incubation of the cultures.

In my earlier experiments the inoculation took place as a rule by transferring a suspension of attacked fibres from a tube with a young culture to the experimental vessels by means of pipettes. Such suspensions

contained c. one million cells per ml¹, and 0.5 ml was generally used as inoculum for each flask. In the course of the investigation it was, however, found that a strong suspension of cells was markedly better than a weaker one, the reason for this perhaps lying in the greater amount of growth factors supplied with the inoculum in this way. Through this a uniform growth was achieved in all parallel flasks. On the other hand, with dilute suspensions used as inoculum the first visible growth was very retarded and appeared very unevenly in the different vessels. This was observed especially when using cotton-wool as a substrate. By using greater concentrations of cells (200—400 millions per ml) these disadvantages were eliminated. Therefore in most later experiments the inoculation has been made by transferring from usual Erlenmeyer flasks with cotton-wool (c. 7—14 days old) which exhibited strong *Cytophaga* development. 0.5—1 ml of the turbid but fibre-free liquid in such flasks, taken out aseptically, was used as inoculum. Visible growth was in this way frequently obtained in the inoculated vessels already in 2 days.

C. Determinations of Residual Cellulose.

To estimate the bacterial growth the most direct method is to count the cells in a counting chamber. The quantity of bacteria present in the cultures might also be measured, for instance, by determining the organic nitrogen or phosphorus. I have preferred, however, to express the development of the bacteria concerned in terms of their metabolic activity and to use the amount of decomposed cellulose as a standard. From a physiological point of view this seems fully justified and it has the advantage of simplicity. The cellulose determinations in cultures of cellulose-decomposing bacteria — *Cytophaga* or others — offer no real difficulties, if only a convenient filtering equipment and a suitable washing procedure for the cellulose are used. The only drawback is sometimes the copious slime formation, which makes filtration difficult. Dissolution and precipitation of the cellulose does not seem to be necessary, as the salts and metabolic products can be easily washed out.

On the other hand, the counting of cellulose-decomposing bacteria to a large extent attached to the cellulose fibre, and held together by slime, is a matter of great difficulty. A further disadvantage when counting the bacteria in question is their extremely low refractivity, which makes the use of light-field illumination almost impossible for this purpose; dark-field

¹ Counted in a Bürker chamber with dark-field illumination. The microcysts were not counted.

illumination by means of a Zeiss "Präparier-Wechsel-Kondensor" has been used by me in some attempts to estimate the number of cells.

In preliminary experiments it was found that a satisfactory washing was effected if the cellulose was treated with acid, alkali and alcohol. To remove the undissolved salts an addition of acid is necessary. This has a further advantage, namely a flocculation of the mucilage formed in *Cytophaga* cultures, which makes the filtration easier. For a complete dissolution and elimination of the mucilage a treatment with alkali seems to be necessary. For this purpose ammonia was used with good results. The alcohol removes the yellow pigment and the water from the cellulose. The ether treatment promotes a rapid drying.

For filtering the cellulose, glass crucibles with fritted glass bottoms of medium porosity have been found very convenient and have been employed throughout the investigation. In most cases Jena crucibles 1 G 3 have been used. A set of 10—12 such crucibles — connected to a filter pump — could easily be handled simultaneously.

The following procedure is now employed in the determinations of the cellulose residue. (This washing procedure is only a slight modification of that used by BENGTTSSON (1925) in determinations of cellulose in soil.)

1. The culture liquid is filtered through the crucible under suction. If the filtrate is not to be used for further studies, the sample is conveniently acidified before filtration.
2. The crucible is filled with 12 % HCl and the mixture is stirred. The liquid is sucked off.
3. The cellulose is washed with water.
4. The cellulose is washed with 5 % ammonia. If necessary (old cultures with large amounts of mucilage) twice.
5. The cellulose is washed with water.
6. The cellulose is washed with alcohol twice.
7. The cellulose is washed with ether and sucked dry.
8. The cellulose is dried at 50° C for one hour and at 103° C for one hour.
9. The cellulose is allowed to cool in a desiccator over granulated CaCl_2 or silica gel.

If cotton-wool is used as a substrate, the cellulose can be transferred from the crucible to a weighing bottle after the ether treatment. It is dried in the bottle and can be picked up and laid directly on the balance-pan at the weighing. In this way no previous weighing of the crucibles or weighing bottles is necessary. By using cellulose-wadding or other sources of cellulose this, again, is inevitable, since the fibres adhere to the disc of the crucible, and thus a complete transference of the cellulose from the crucibles

is difficult. The use of cotton-wool in the experiments consequently offers a great advantage, less time being required for the determinations.

At the weighing an accuracy of ± 1 mg was considered sufficient, as the variation between parallel samples is in general much greater.

It has been verified that the residual cellulose, determined in this way, corresponds to the real residue from a chemical point of view. It is, in fact, possible that bacterial cells and metabolic products adhere to the fibres and are not completely removed, thus giving too high values for the cellulose residue. For that reason in an experiment the washed cellulose was dissolved in Schweizer's reagent and precipitated from the solution with alcohol, according to the modified method of CHARPENTIER (1920, see also LINDBERG 1944 p. 19).

By this method it was demonstrated that only slight and insignificant differences existed, not amounting to 1 per cent of the weight of the cellulose. Samples with a varying degree of decomposition were analyzed.

Another point should also be taken into consideration. The crucibles used have a porosity of 15–40 μ . Thus particles with smaller dimensions are not retained. If the cellulose decomposition proceeds in such a way that fractions are formed which chemically exhibit the properties of cellulose but are small enough to pass through the crucibles, this must give a false idea of the extent of the decomposition. It may be noted in this connection that MEYER (1943) observed a great many microscopic fragments of fibres in cultures of anaerobic cellulose-decomposing bacteria. In *Cytophaga* cultures, however, the quantity of such fragments seems to be quite negligible. In order to verify this point, determinations of cellulose were made in the filtered culture liquid.

The filtered cultures were evaporated to dryness and the residue was treated with Schweizer's reagent. The determination was then carried out according to the above-mentioned method. Although samples with a varying degree of cellulose decomposition were examined in this way, in no case could any cellulose be recovered from the filtrates.

The conclusion is to be drawn that, if the cellulose is broken up into fragments still of a cellulosic nature but too small to be retained by the crucibles used, such fragments must be immediately further used up by the bacteria. They cannot be detected in cultures of living bacteria.

III. Culture Media.

A. The Nutrient Solution.

As frequently demonstrated, *Cytophaga* is able to grow at the expense of cellulose in an entirely inorganic medium. I have myself made only a few accurate experiments concerning the composition of the nutrient media; some observations made during the course of the investigation are recorded in the following pages.

1. Composition of the mineral base.

At an early stage of the present investigation various mineral solutions recommended for cellulose-decomposing bacteria were compared. The main divergence between these media seems to be the different source of nitrogen, but the content of trace elements may also vary. Among others the media used by OMELIANSKI (1902), HUTCHINSON and CLAYTON (1919) and WAKSMAN and CAREY (1926) were tested in tubes with filter paper as substrates for *Cytophaga* 3. The composition of these media is as follows:

OMELIANSKI:	
(NH ₄) ₂ SO ₄	1.0 g
K ₂ HPO ₄	1.0
¹ MgSO ₄	0.5
NaCl.....	traces
aq. dest.	1000

WAKSMAN and CAREY:	
(NH ₄) ₂ HPO ₄	2.5 g
¹ MgSO ₄	0.5
¹ FeSO ₄	0.01
KCl.....	0.5
aq. dest.	1000

HUTCHINSON and CLAYTON:	
KH ₂ PO ₄	1.0 g
CaCl ₂	0.1
MgSO ₄ · 7 H ₂ O.....	0.3
NaCl.....	0.1
Fe ₂ Cl ₆	0.01
NaNO ₃	2.5
aq. dest.....	1000

WAKSMAN and CAREY, modified (see below):	
(NH ₄) ₂ HPO ₄	2.5 g
MgSO ₄ · 7 H ₂ O.....	0.5
FeSO ₄ · 7 H ₂ O.....	0.01
KCl.....	0.5
CaCl ₂	0.02
MnSO ₄ · 4 H ₂ O.....	0.001
aq. dest.....	1000

¹ Crystal water not given. I used the salt with 7 H₂O.

All media were adjusted to pH 7.4 with NaOH.¹

The experiment, being only qualitative in nature, did not reveal greater differences in the suitability of the media. At a later stage the medium of OMELIANSKI seemed to be more unfavourable. This is possibly a pH effect, since the medium contains $(\text{NH}_4)_2\text{SO}_4$ and the pH will sink very markedly at the absorption of the ammonium ion. Of the other two media, one containing ammonium and the other nitrate as source of nitrogen, the medium indicated by WAKSMAN and CAREY was simpler to prepare. It offered the advantage of having a great buffer capacity through its high content of ammonium phosphate. See also BUCKSTEEG (1936 p. 8), who compared various nitrogen sources for a *Cytophaga* strain.

The medium of WAKSMAN and CAREY was then used as basal medium in most experiments, as the growth of all *Cytophaga* strains studied was very good on this substrate. Thus *Cytophaga globulosa* grew better in this medium than in the "A-Lösung" indicated by STAPP and BORTELS (1934). This may be due to differences in the tap water of Berlin and Uppsala respectively, since STAPP (personal communication) reports a better growth of *C. globulosa* on "A-Lösung".

Certain results obtained later caused a modification of this medium. It became evident that calcium and manganese, which were not present in the original medium, were, if not necessary, at least stimulating in the cultivation of *Cytophaga* 3. Accordingly, a modified medium containing Ca and Mn was made up (see page 21). This medium, simply called Waksman's medium in the subsequent pages, has been used in most quantitative experiments reported in this paper.

The addition of Ca and Mn did not appear to have any significance in experiments with filter paper as a source of cellulose. The very purest, ash-free qualities also, e. g. Munktell no. 00 or Schleicher & Schüll no. 589, could be used as substrates without an addition of Ca or Mn, the growth of *Cytophaga* being equally good without as with the addition of these metals. But in experiments with cotton-wool as a source of cellulose these elements were found strongly to stimulate the decomposition of cellulose. In certain experiments no growth at all was observed on cotton-wool, if no Ca was added to the medium. Thus *Cytophaga* 3 appears to require Ca in small quantities, although this is apparent only when using an extremely pure cellulose.

The need for Mn was, on the other hand, less clear. An addition of 0.1 mg $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ per litre (= 1 γ /tube) greatly stimulated the growth on cotton-wool in certain experiments, although growth occurred

¹ All pH values given in the present work are determined electrometrically, unless otherwise stated. In most cases a Beckman potentiometer with a glass electrode was used.

also without any addition of Mn. This may be due to a certain content of Mn in the cotton or chemicals used. A higher concentration than 0.1 mg/lit. did not increase the effect further.

2. pH conditions.

In connection with the nutrient medium used some words should also be said about the pH conditions. The Waksman solution used throughout the study has a pH value of about 7.0 after sterilization. This value lies within the optimal pH range. In the course of cellulose decomposition the pH of this solution decreases and becomes more unfavourable after passing about 6.5. On the other hand, a nitrate medium increases its pH value in the course of the cellulose decomposition. It consequently at last also becomes unfavourable. The upper limit for growth seems to lie already at about pH 8.5.

The pH conditions have been subjected to an experiment, in which the nitrogen source was ammonium phosphate. Organic nitrogen sources were tried but proved unsuitable. It does not seem possible to use such substances, when working with these bacteria. Otherwise it would be very convenient, since the pH under such circumstances does not change to such an extent as with inorganic nitrogen sources.

The medium used in the experiment was Waksman's solution with 1.32 g (M/100) ammonium phosphate per litre and with cellulose-wadding as source of cellulose. The pH was adjusted by a further addition of phosphate buffer. The final phosphate concentration in the experiment flasks was M/50. A stronger solution seems to inhibit the growth or at least retard it considerably.

Varying pH values were obtained by addition of mixtures of HCl, KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and K_3PO_4 . This addition was made aseptically with tenfold concentrated (M/10) solutions to the 90 ml of medium. Regarding the pH determinations, compare footnote 1, p. 22. Values between 5.0 and 9.0 were tested. The results will be seen in the accompanying Table (Table 1) and Figure (Fig. 1).

As seen from the Table and the Figure the growth in the beginning proceeds most rapidly at pH 7.4—7.7 (original values). At 8.3 the growth is almost absent and at pH 7.0 the development is somewhat weaker than at pH 7.4—7.7. After one month's culture the conditions are on the whole the same. The greatest decomposition values are now found in the series originally having pH 7.4—8.3, the flasks with the original pH value of 8.3 being now within the optimal range (7.3). Even at the low pH of 5.9 a certain decomposition of the cellulose took place in some flasks. The very uneven growth shows that the lower limit for growth is approached here. At pH 5.0 and 9.0 no growth occurred.

With *Cytophaga globulosa* no particular pH experiment was carried out. STAPP and BORTELS (1934) state that the optimum lies about the neutral point, the minimum at about 6.0. This agrees with my experience.

Table 1. pH Experiment with *Cytophaga* 3.

Original Cellulose Quantity: 517 mg. Temperature 25° C.

Original pH	8.3	7.7	7.4	7.0	6.4	5.8
<i>7 days:</i>						
Cellulose recovered mg/flask ..	509	443	451 ¹	468	487	
	517	450		462	497	
	519	452	455 ¹	461	496	
		461		467	498	
		459	459 ¹	461	498	
		462		463	495	
Mean values.....	515	455	455	464	495	
Cellulose consumed.....	2	62	62	53	22	—
Final pH	8.0	7.3	7.1	6.8	6.4	
<i>30 days:</i>						
Cellulose recovered mg/flask ..	330	333	349	353	372	400
	348	336	326	368	371	416
	331	330	336	349	370	439
	327	326	349	356	379	465
	347	325	348	347	374	
	340	340	351	358	376	
Mean values.....	337	332	343	355	374	
Cellulose consumed.....	180	185	174	162	143	117-52
Final pH	7.3	7.1	6.9	6.6	6.0	5.9-5.6

¹ 2 parallel samples combined.

3. Growth factor requirements.

In connection with the nutrient media a few words should be said about the growth factor requirements of *Cytophaga*. Until quite recently no investigations had been published concerning this problem. Last year a study partly dealing with this subject was published by HARMSSEN (1946). However, he has not tested the pure substances, but has only studied the influence



Fig. 1. pH experiment with *Cytophaga 3*. A: cellulose decomposed after 7 days.

B: cellulose decomposed after 30 days.

of various extracts and examined the symbiotic relationships between different cellulose-decomposing bacteria and pointed out some interesting aspects concerning these matters.

I have myself made only a few experiments regarding thiamin. An experimental series was first set up in order to examine whether thiamin was perhaps produced by *Cytophaga*.

For this purpose *Cytophaga 3* was cultured in aerated Erlenmeyer flasks with 0.5 per cent filter paper (Munktell no. 0)¹ in Waksman's medium. One flask was withdrawn every day for a week and the filtrate preserved in a refrigerator. The thiamin content was then tested in an experiment by the *Phycomyces* method. The absence of contaminating microorganisms in the culture filtrates was proved.

As a nutrient medium for *Phycomyces* SCHOPFER's solution (see FRIES 1938 p. 128) was employed; it contains glucose 5 g, asparagin 1 g, KH_2PO_4 1.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g and aq. dest. 1000 ml.

¹ Finely divided in a dry defibrator.

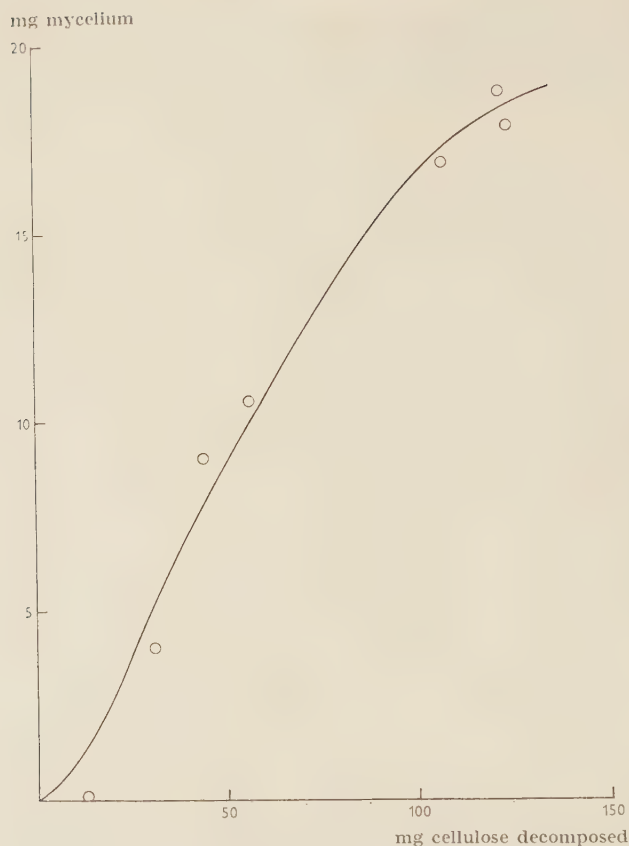


Fig. 2. Growth of *Phycomyces Blakesleeanus* on culture filtrates of *Cytophaga* 3.

100 ml Erlenmeyer flasks, each containing 25 ml of the solution, were used. 2 ml filtrate¹ of the *Cytophaga* cultures were added to the basal medium in each flask. 4 parallel flasks with each filtrate and, in addition, controls with pure thiamin and without any addition were set up. The flasks were inoculated with a spore suspension of *Phycomyces Blakesleeanus* (—). After 9 days the mycelia were collected, washed in water, dried and weighed.

The cellulose decomposition was determined in those flasks from which the filtrates were taken out.

From the fact that *Phycomyces* developed readily in the flasks it was evident that thiamin had been formed to a considerable extent in the *Cytophaga* cultures. The amounts of *Phycomyces* mycelium produced in 9 days are plotted in Fig. 2 as functions of the values for decomposed cellulose in the *Cytophaga* cultures from which the filtrates were taken. The addi-

¹ Not heated and thus containing living bacteria. These do not develop, however, at the low pH value in the flasks.

tion of 10 mγ pure thiamin gave a mycelium quantity of 11.8 mg. In the control flasks without any addition less than 0.02 mg mycelium was formed.

From the Figure it will be seen that the formation of thiamin in the *Cytophaga* cultures is fairly proportional to the cellulose decomposition, at least in its first stage.

From the fact that *Cytophaga* itself is able to produce thiamin, it is not to be expected that an addition of that substance is necessary when cultivating *Cytophaga*, although thiamin is probably necessary for its metabolism. However, it was possible to show that thiamin was in fact a stimulating agent when added to tubes which are inoculated with very dilute suspensions of cells.

In such an experiment culture tubes containing 10 ml of Waksman's medium with a strip of filter paper (Munktel no. 00, see also below p. 31) were used. Thiamin solutions of varying strength were autoclaved separately¹; 1 ml containing 1, 10 and 100 mγ respectively was added to the tubes. Tubes with the addition of culture filtrate of *Cytophaga* 3 and without any addition were also set up. The inoculum contained about 1000 *Cytophaga* cells (see footnote, p. 18). 4—6 parallel tubes were inoculated. Table 2 shows the result of this experiment.

Thus the attack on cellulose was obvious after 3 days, if 0.1 γ thiamin was added but not until after 6 days in the controls without thiamin and then not in all tubes. With culture filtrate growth was obvious after 3—4 days and it seems probable that this is at least partly a thiamin effect.

The filtrates apparently also contain at least one additional substance of vitaminous nature, namely a substance promoting germination of *Phycomyces* spores, but these matters have not been studied in more detail. The evaluation of results must be very cautious, since even "pure" cellulose may contain such substances (ROBBINS and SCHMITT 1945).

Other experiments, where thiamin has been added to the medium, have not given the same positive evidence for the necessity of thiamin. In a later experiment with *Cytophaga* 3 no positive result was obtained. Possibly the inoculum was too large and thus sufficient quantities of thiamin were added in this way.

It is probable that the growth factor requirements of different *Cytophaga* strains vary.² In this very respect a divergence may exist between the microcyst-forming and the amicrocystogenous types within the *Cytophaga* group (cf. p. 11). Evidence in this direction is given by STAPP and BORTELS (1934), who found that the non-microcyst-forming species *Cytophaga silvestris*, *anularis*, *crocea* and *flavicula* were not able to grow in pure culture.

¹ At pH 5; a partial destruction of the thiamin might have occurred, however.

² In this connection the results of NILSSON *et al.* (1939) with various strains of *Rhizobium* should be observed.

Table 2. Effect of the Addition of Thiamin on the Growth of *Cytophaga* 3.

Addition		Results after hours:				
		72	78.5	84.5	96	144
None	1-4	—	—	—	—	—
	5-6	—	—	—	—	+
1 mγ thiamin	1	—	—	—	+	++
	2	—	—	—	+	++
	3	—	—	—	+	++
	4	—	—	+	+	++
10 mγ thiamin ...	1	—	—	+	+	++
	2	—	+	+	+	++
	3	—	—	—	+	++
	4	—	+	+	+	++
100 mγ thiamin ..	1	+	+	+	+	++
	2	+	+	+	+	++
	3	+	+	+	+	++
	4	—	+	+	+	++
Culture filtrate ...	1	—	—	—	+	++
	2	—	+	+	+	++
	3	—	—	+	+	++
	4	—	+	+	+	++

— no growth.

+ faint but obvious yellow points of attack on the paper.

++ strong development (yellow zone on the paper at the liquid surface).

This fact might well be explained by assuming that some vitamins are necessary for growth and that the species in question have lost their ability of synthesizing them. They are consequently dependent on "symbiotic" organisms for growth, unless the factors required are recognized and added to the medium at cultivation. It must, however, be admitted that STAPP and BORTELS's results so far have not been confirmed by other authors.

HARMSSEN (1946 p. 185), for instance, who studied the symbiotic relationships between *Cytophaga* and other microorganisms and found a considerable stimulation of *Cytophaga* through the action of *Bacillus* species, has not observed any differences in the behaviour of the microcyst-forming and non-microcyst-forming strains, respectively. However, there are somewhat contradictory statements on pp. 91 and 185 in his publication.

In *Cytophaga* I have myself observed a stimulation by contaminating bacteria in many cases. This is only a confirmation of the earlier findings

of IMŠENECKI and SOLNCEVA (1936). STANIER (1942 b), however, claims that he has never observed a stimulatory effect of this nature (p. 169).

It is clear that a stimulation by foreign bacteria is not necessarily due to growth substances. Very likely the accompanying bacteria are able to improve the conditions in some other way also, for instance through removing unfavourable metabolic products or changing pH in a suitable direction. Compare also p. 31 below.

B. Sources of Cellulose.

In the course of the investigation various forms of cellulose have been used. Some types have been used only for special purposes. For most growth experiments, however, two kinds of cellulose have been employed: cellulose-wadding (Zellstoff) and cotton-wool. Because of their suitable physical consistence, they are easy to disperse evenly in a liquid medium. Chopped or ground filter paper, on the contrary, very soon sinks to the bottom of the culture flasks. The purest available qualities of both substrates were used. The cellulose-wadding was manufactured by Långasjönäs A/B, Kisa; the cotton-wool was commercial high grade cotton, "charpi-bomull".

In the beginning of the present investigation the best results were obtained by using cellulose-wadding. Determinations of decomposed cellulose agreed very well in parallel flasks with this substrate, whereas cotton-wool did not give such good parallel values. In many cases the growth was very scanty and irregular on cotton. This was probably due to lack of purity of the cotton. When this became clear and carefully selected cotton was used, values with fairly good agreement were obtained. Consequently, cotton-wool has been employed in most experiments since 1941.

In this connection it may, however, be noted that many cellulose-decomposing bacteria (*Cellvibrio*, *Corynebacterium*) have not been able to utilize cotton-wool as a source of cellulose in my experiments, although cellulose-wadding has been readily utilized. This point is difficult to explain. All strains grow, in fact, very well on filter paper, and this is, as we know, made from cotton fibres or linen. It seems somewhat remarkable that cotton-wool is not attacked under these circumstances. The cellulose-wadding used is, on the contrary, made from sulphite cellulose. Possibly some essential factor of vitaminous nature present in filter paper and cellulose-wadding, but not in pure cotton-wool, is required by the bacteria mentioned. According to HARMSSEN (1946) *Cellvibrio*, for instance, is more dependent on accessory growth factors than is *Cytophaga*.

An advantage of cotton-wool as compared with cellulose-wadding is its purity. The cotton-wool used by me contained 98—99.5 % α -cellulose, whereas the cellulose-wadding contained only 85—90 % α -cellulose. The determinations of α -cellulose were carried out according to RITTER (1929, method 4). A determination of cellulose content with Schweizer's reagent (CHARPENTIER's method, see above p. 20) yielded about 95 % cellulose in the cellulose-wadding used. The non-cellulosic matter is perhaps pentosanes but has not been examined by me. It is of interest to note that the very purest quality of Swedish filter paper (Munktell no. 00) seems to contain only between 90 and 95 % α -cellulose, while the less pure makes (e. g. nos. 0 and 3) contain about 95 %. This is probably due to a certain degradation to low molecular cellulose during the purification in the factory.

When using cellulose-wadding as a substrate, logically a correction should have been made for the non-cellulosic matter at the calculation of the experimental values. This has, however, not been done, since an adequate correction would have required a great many additional analyses of the residual substance and since in any case the difference between the corrected values and the given values does not seem to be important.

For special purposes precipitated cellulose, cellophane, lichenin, etc., have been used. These substrates will be described in greater detail in connection with the respective experiments.

One particular point concerning the source of cellulose is important when working with *Cytophaga*. As will be discussed later, the bacteria here referred to are very sensitive to even small concentrations of soluble organic substances (see p. 34). It is evident from my experiments that such substances are present also in celluloses of various kinds. That means that a large concentration of cellulose often exhibits an unfavourable effect through the content of inhibiting substances. When cultivating *Cytophaga* in liquid cultures, for instance, 0.5 % cellulose-wadding is decidedly more suitable than 1 %, but even 0.5 % is to some extent inhibitive. By using agar plates with filter paper placed on the surface, the inhibitory effect is very apparent. A 9 cm filter paper placed in a Petri dish with 10 ml agar gives a concentration of about 5 % cellulose. This concentration entirely inhibits the development of *Cytophaga*. The inhibition is not caused by lack of moisture, which can be shown by total saturation with liquid. The concentration of agar (1.5 %) cannot in this case be of importance (cf. above p. 14), since the cellulose is not immersed in the agar, and the agar was previously extracted in order to eliminate inhibitory substances (see p. 70). By using strips of paper, weighing only 0.05—0.1 g for each plate (0.5—1 %), a fairly good growth was observed. The concentration allowed varied in respect to the make of paper used.

If the agar was not previously purified through extraction no growth occurred even on the plates with only 0.05 g filter paper.

It was observed that when growth occurred on plates containing 0.2 g (= 2 %) or more of cellulose, these plates were in general infected by contaminating bacteria or moulds. The contaminant developed first, and then the yellow zones of *Cytophaga* became evident. This may also be an indication of the presence of inhibiting substances in the papers. If the substances in question are soluble organic compounds, they will very probably be utilized by several versatile organisms and thus be made harmless to *Cytophaga*.

This fact may partly explain the experiences of many authors that the cytophagas are difficult to obtain in pure culture free from other bacteria and that certain species may need some kind of symbiosis. Through their sensitivity to even small concentrations of certain soluble substances they will generally grow best in the presence of organisms which are able to remove these substances.

It is possible to extract the inhibiting substances from the cellulose, at least partly. Experience showed that alkaline solutions were favourable for this extraction. Also by treating the cellulose with the usual, although somewhat simplified¹, mineral medium, a fairly good extraction was achieved. The method generally employed was the following. 1 g filter paper was treated with 100 ml solution in an autoclave at 120° C for 30 minutes. Subsequently the papers were washed carefully in water and dried at 50° C. Strips were then cut and sterilized in an autoclave at 120° C. They were placed aseptically on poured agar plates with purified 1.5 per cent agar, containing the necessary salts. The strips were finally inoculated with 0.5 ml suspension of *Cytophaga*.

The inhibiting substances are not confined to some particular kinds of filter papers. An experiment, the results of which are given in Table 3, showed that these substances are present in different makes. In all cases the inhibitory effect could, at least partly, be removed by treating the papers in the manner indicated above.

The papers used were Munktell no. 10: unwashed², 0 B: single-washed, 00: double-washed and, further, Schleicher & Schüll 589—2, double-washed, the two last-mentioned having a very low ash content.

It will be seen from the Table that the Schleicher & Schüll paper contains the inhibiting substances to an even higher degree than does the 0 B paper, for instance. Unfortunately I have had no opportunity to examine the English Whatman paper, which has been much employed by various investigators in experiments with cellulose-splitting bacteria.

¹ Containing only $(\text{NH}_4)_2\text{HPO}_4$, KCl and MgSO_4 .

² This refers to the treatment in the factory. Single-washed: treated with HCl, double-washed: treated with HCl and HF.

Table 3. Growth of *Cytophaga* 3 on Agar Plates with Untreated and Extracted Filter Papers. 2 Parallels.

Amount of cellulose	Growth of <i>Cytophaga</i>											
	0.05 g			0.1 g			0.2 g			0.4 g		
Days	4	6	14	4	6	14	4	6	14	4	6	14
<i>Filter paper:</i>												
10 untreated	1	2	3	1	2	3	—	1	1	—	—	1 ¹
10 extracted	1	3	3	2	3	3	1	3	3	1	2	2
0 B untreated	1	2	3	1	3	3	—	1	2	—	1	1
0 B extracted	2	3	3	1	3	3	1	2	2	1	2	2
00 untreated	1	3	3	—	1	2	—	1	2	—	—	—
00 extracted	—	1	2	1	2	3	—	1	2	—	1	2
589 untreated	1	1	3	—	1	2	—	—	—	—	—	1 ¹
589 extracted	—	1	3	1	2	3	1	1	3	—	1	2

— no growth.

3 maximal growth.

It might be suspected that the inhibiting agents were formed by autoclaving the papers from originally harmless substances present in the paper, by analogy with the unfavourable effect on glucose through heating (cf. p. 40). For practical use the heat sterilization cannot, however, be avoided. In order to examine the possibility mentioned, I tried a sterilization with alcohol, since this disinfectant could be evaporated after the treatment, without any substances being extracted. From the result of this experiment it seems probable that the inhibiting substances are already present in the papers before the heat sterilization.

Regarding the nature of the inhibiting substances, nothing is known so far. I have tried to concentrate these substances, but without success. It is perhaps not necessary to postulate an organic substance in this connection. Possibly one might suspect a certain content of fluoride or fluosilicate in the papers, which, as we know, are washed with HF in the factories when preparing the purest qualities. These qualities are also generally those most resistant to attack of the bacteria concerned.

In some experiments I have demonstrated that *Cytophaga* is very sensitive to fluoride, a concentration in the nutrient medium of even 0.001 % NaF being unfavourable. The very low ash content of the purest filter papers seems, however, to exclude the possibility of fluorides being the inhibiting agents.

¹ In these cases a development of contaminating moulds or bacteria was observed before any growth of *Cytophaga* took place.

The occurrence of inhibitory substances in various kinds of filter papers may be of secondary interest only. However, this fact has caused very much trouble, especially at an early stage of the present investigation, since nothing was previously known on this subject.

The fact that usual filter papers contain these inhibiting substances may possibly have a wider bearing. Filter papers are widely used as a substrate for germination in the study of seedlings of higher plants. It is to be expected that the germination process in certain cases might be sensitive to the substances here referred to. These substances would then interfere with the results of such studies.

IV. Influence of Soluble Carbohydrates on the Growth and Cellulose Decomposition by *Cytophaga*.

A. Review of the Literature.

HUTCHINSON and CLAYTON (1919) noted as one of the most outstanding features of "*Spirochaeta cytophaga*" the marked specialization in its nutritive requirements. They add: "at the same time it displays extraordinary sensitiveness towards the presence of compounds which it is apparently unable to utilize but which with the general run of bacteria have been found to serve as sources of nitrogen or carbon or both — peptone, amino-acids, sugars, salts of organic acids, etc."

Especially the reducing sugars were found to be extremely inhibitive, irrespective of the presence or absence of cellulose. The general method employed in these studies was the estimation of growth in tubes containing nutrient solution with sugar and a filter paper strip partly immersed in the solution.

HUTCHINSON and CLAYTON found that, for instance, glucose is markedly inhibitory even in a concentration of 0.01 %. The following Table, taken from their paper (1919, p. 161), gives a good picture of the facts:

Table 4. Inhibitory Effect of Dextrose on the Growth of *S. cytophaga* in the Presence of Cellulose.

Growth after	Concentration of solution (per cent)						
	Control	0.005	0.01	0.025	0.050	0.10	0.25
3 days	++	++	-+	-+	-	-	-
8 days	+++	+++	+++	-+	-+	-	-
12 days	+++	+++	+++	+++	++	-	-

Maltose was found to be even more toxic, a 0.018 per cent solution being strongly inhibitory and a 0.037 per cent solution completely inhibitory to growth. Of the other reducing sugars, arabinose, fructose and galactose were inhibitory in a 0.1 % concentration (lower concentration not tested), while lactose in this concentration permitted a faint development. The non-reducing sugars, saccharose and raffinose, starch, and the alcohols, mannitol, adonitol, and dulcitol, were all tolerated in 1 % solutions. In no case was any growth observed at the expense of the sugars added.

Their experimental results logically led the authors to the assumption of cellulose being the only utilizable carbon compound for *Spirochaeta cytophaga*.

These findings were later confirmed by all investigators up to recent years. All authors have drawn the conclusion from their results that *Cytophaga* cannot utilize other sources of carbon than cellulose (DUBOS 1928, WINOGRADSKY 1929, BOKOR 1930, STAPP and BORTELS 1934, BUCKSTEEG 1936, IMŠENECKI and SOLNCEVA 1936, WALKER and WARREN 1938, JENSEN 1940, IMŠENECKI 1941).

The critical range of glucose and other sugars given by these authors varies, however, within certain limits. Thus according to HUTCHINSON and CLAYTON maltose prevents growth entirely at 0.037 %, but according to IMŠENECKI (1941) first at 0.8 %. Cellobiose was found to be inhibitive at 0.1 % (*Cytophaga silvestris*) by STAPP and BORTELS, but first at 1.0 % by WALKER and WARREN. Glucose seems in general to be the most toxic of the sugars tested.

The complete unsuitability of carbohydrates other than cellulose thus seemed to be fully ascertained through the accumulated experimental evidence. This fact was indeed very remarkable. The various pentoses and hexoses not closely related to cellulose may well be thought unsuitable for growth, but the building units of the cellulose molecule, cellobiose and glucose, should for an unprejudiced mind be attacked as equally well as cellulose.

It is, however, to be observed that quantitative determinations of the sugars examined were never made. With the Myxobacteria *Sorangium compositum* and *S. nigrescens* quantitative studies of the cellulose and glucose metabolism have been made to some extent by H. and S. KRZEMIENIEWSKI (1937), but not with *Cytophaga*. The statements given by various authors were based solely on subjective observations of the growth in tubes containing strips of filter paper and the respective sugar to be tested.

The attack on the cellulose can, of course, be easily observed in this way. But the statements regarding utilization of the sugars and limit concentrations for growth must always be a matter of some doubt, if no sugar determinations are made. If very small amounts of sugar are used, these are perhaps totally used up previous to the attack on the cellulose. The observation of HUTCHINSON and CLAYTON and others that 0.05 % glucose is first inhibitory but later allows as strong a development of *Cytophaga* as in control tubes without glucose, may be interpreted in this way. A consumption of very small quantities of glucose is possibly not evident as an obvious turbidity of the medium.

The uncertainty of such statements is especially manifest in cases where the *Cytophaga* species studied is accompanied by some "symbiotic" bacteria, as, for instance, in some of the strains of STAPP and BORTELS (1934) or the strain used by WALKER and WARREN (1938). These "symbionts" may well utilize glucose and thus interfere with the experimental results. The "symbionts" of STAPP and BORTELS could in fact be cultivated on 0.5 % glucose agar (l. c. p. 34).

Recently it was in fact shown independently by FÄHRÆUS (1941) and STANIER (1942 a) that glucose and cellobiose could be utilized as sources of carbon by *Cytophaga*, if only small concentrations of the sugar were used (FÄHRÆUS) or if the sugars were sterilized by filtration instead of being autoclaved (STANIER). Our results have since been confirmed by others, for instance by HARMSSEN (1946).

A report will be given here of my own experiments regarding the suitability of the soluble carbohydrates, especially glucose. Regarding the results of STANIER (1942 a, b) see also p. 53. In the essential point, namely the utilization of glucose as a source of carbon, our results are in full agreement. The cause of the inhibitory effect is still a matter of some confusion, and this problem will be discussed in more detail later (p. 52).

B. Qualitative Experiments.

My first experiments were carried out with the aforementioned strain 3. This newly isolated strain was tested on a number of soluble carbohydrates. The influence on the cellulose decomposition was studied in the usual manner in tubes with nutrient solution and filter paper strips (Munktell no. 3).

The sugars were autoclaved separately (10 per cent solutions) and added aseptically to the tubes (cf. p. 40). In the fructose and dextrin solutions a certain caramelization took place.

The result of this qualitative experiment is given in Table 5.

These results agree on the whole with earlier statements. Possibly the sensitivity towards sugars is somewhat lower than that previously reported for other *Cytophaga* strains. But regarding glucose there exists a remarkable difference. Low concentrations up to 0.1 % did not have any marked influence on the cellulose decomposition. The bacterial development in these tubes appeared to be very vigorous and the colour was more intense than in the control tubes. 0.5 and 1.0 % glucose initially inhibited the growth and cellulose decomposition totally. Later, after 7 days, minute yellow points appeared on the paper at the liquid-air interface. These

Table 5. Influence of Carbohydrates on the Decomposition of Cellulose by *Cytophaga* 3.

Sugar added ¹		Growth on cellulose after days				Visible growth on sugar (10 days)
		2	3	5	21	
No addition		+	++	+++		
Xylose	0.01 %	+	++	+++		—
	0.1	—	++	+++		—
	1.0	—	—	+	++	—
Fructose	0.01 %	—	++	+++		—
	0.1	—	—	++		—
	1.0	—	—	—	—	—
Glucose	0.01 %	+	++	+++		—
	0.05	+	++	+++		—
	0.1	+	++	+++		—
	0.5	—	—	—		++
	1.0	—	—	—		++
Galactose	0.01 %	+	++	+++		—
	0.1	+	++	+++		—
	1.0	—	—	—	+++	—
Lactose	0.01 %	+	++	+++		—
	0.1	+	++	+++		—
	1.0	+	++	+++		—
Maltose	0.01 %	+	++	+++		—
	0.1	+	++	+++		—
	1.0	+	++	+++		—
Sucrose	0.01 %	+	++	+++		—
	0.1	+	++	+++		—
	1.0	+	++	+++		—
Dextrin	0.01 %	+	++	+++		—
	0.1	+	++	+++		—
	1.0	—	+	+++		—
Starch	0.01 %	+	++	+++		—
	0.1	+	++	+++		—
	1.0	—	++	++		—

— no growth.

+++ maximal growth.

¹ The sugars employed in this and the following experiment (p. 38) were: l(+) arabinose "Merck", xylose "rein Kahlb.", fructose "reinst Kahlb.", glucose "puriss. anhydr. DAB 6 Kahlb.", mannose "Merck", galactose "Kahlb.", cellobiose "Fränckel & Landau", lactose "für bakteriologische Zwecke, Kahlb.", maltose "reinst Kahlb.", sucrose "analar", raffinose "n. Ritthausen, Merck", dextrin "Merck", starch "soluble, analar".

points of attack later formed larger, raised, yellow colonies on the paper but did not cause the usual weakening and breaking up of the paper. Nor was the paper itself yellow-coloured. Simultaneously the solution became considerably turbid. Gradually the colonies covered the whole paper in the surface zone, but the paper was not attacked.

In the experiment reported above the influence of various sugars was studied in the presence of cellulose. A second experiment was then set up, in order to examine the influence of sugars without any cellulose being present. In the first experiment the cellulose, so to speak, formed a substrate for the bacterial growth also in case the cellulose was not attacked (colonies on the paper in the glucose series!) and the aerobic requirements seemed satisfied in this way. In the experiment without cellulose it seemed more convenient to use flasks with a shallow layer of sugar solution. Consequently 100 ml Erlenmeyer flasks with 10 ml Waksman's medium containing 0.5 % of the respective sugars were used. The flasks were inoculated with a cell suspension of *Cytophaga* 3, carried on glucose. The sugars were sterilized as in the preceding experiment.

The result is given in Table 6.

From this Table it is evident that not only glucose but also cellobiose and mannose are suitable as sources of carbon. The other sugars appear to be unsuitable.

Table 6. Growth of *Cytophaga* 3 on Various Carbohydrates.

Sugar	Growth after	
	2 days	4 days
Arabinose	—	—
Xylose	—	—
Fructose	—	—
Glucose	+++	+++ r
Mannose	+	++
Galactose	—	—
Cellobiose	+++	+++ r
Maltose	—	—
Lactose	—	—
Sucrose	—	—
Raffinose	—	—
Dextrin	—	—
Starch	—	—

— no growth +++ maximal growth.

The microscopic picture of the bacteria grown on glucose as compared with those grown on cellulose was not essentially changed, but the cells had in general acquired an enlarged and swollen shape, similar to that of the intermediary stages in the formation of microcysts, which can be seen from Pl. II. This shape, characteristic for cultures on glucose, is suggestive of bacterial involution forms, but in this case the reproduction ability does not seem to be diminished.

In studying the literature I found some interesting statements in this connection. STAPP and BORTELS write (1934 p. 54): "Im Hängetrophen mit 0.1 % Tetraose als alleiniger Kohlenstoffquelle wurden teratologische Formen beobachtet, die aussahen wie stark aufgeblähnte und ungewöhnlich grosse Übergangsstadien bei der Mikrocytenbildung." IMŚENECKI and SOLNCEVA report (1936, summary): "Under the influence of sugar the cells change their form and acquire an involutional character." A more detailed study of the changes brought about by glucose addition is given by KRZEMIENIEWSKA (1933). She has studied the germination of the microcysts and writes (pp. 403—4): "Même les sucres . . . n'empêchent pas la germination des microcystes même dans une dilution à 0.5 %. Celle-ci une fois accomplie, l'action des sucres sur les jeunes bâtonnets devient plus ou moins nuisible . . . Seule l'influence du dextrose se manifeste d'une manière différente; les cellules . . . sont plus grandes que d'ordinaire et se transforment en bâtonnets considérablement plus gros ($5-8\ \mu \times 0.5-1.0\ \mu$) avec la chromatine agglomérée soit au milieu, soit à un des deux bouts. . . Le procès de désorganisation ne se fait pas attendre; les bâtonnets s'allongent même jusqu'à $20\ \mu$, subissent des gonflements locaux et leurs contours s'effacent, ce qui constitue le premier symptôme de la décomposition totale."

These observations are in good agreement with the conditions in my strain *Cytophaga 3*. One cannot, however, speak of "décomposition totale". The reproduction is not totally arrested, and from cultures on glucose one can subculture *Cytophaga 3* in numerous generations, without the ability of cellulose decomposition being lost. To maintain such glucose cultures it is, however, necessary to make transfers more frequently. The explanation of this may be the stronger acid formation or some other unfavourable circumstance.

KRZEMIENIEWSKA (1933) did not make any physiological experiments with her *Cytophaga myxococcoides*, unfortunately. From the information given by her one cannot decide, then, whether this strain was capable of using glucose or not. Judging from her morphological findings this does not seem unlikely.

Indisputable information about this point is to be gained only through experiments in which sugar determinations are made. In the following pages a report is given of the experiments carried out in this direction.

C. Quantitative Glucose Experiments.

1. Experimental procedure.

In these experiments as usual 300 ml Erlenmeyer flasks containing 100 ml of Waksman's medium with 0.5 % cellulose were used. The flasks were sterilized in an autoclave at 120° C. In the series with addition of glucose, this was separately sterilized in distilled water under the most considerate conditions possible and added aseptically to the flasks. Thus the autoclaving of the glucose solutions in a phosphate medium was avoided.

The autoclaving was carried out either in a gas-heated autoclave at 115° C for 20 minutes, or in an electrically heated autoclave. In the latter case the heating was turned off when 120° C was reached.

LEWIS (1930) made the observation that inhibiting substances are frequently formed when glucose and ammonium phosphate are heated together. BAUMGARTNER (1938) and DAVIS and ROGERS (1939) examined the influence of autoclaving sugars in distilled water and in the presence of phosphate, and found some sugars, e. g. glucose, to be very sensitive to heating with phosphate. A prolonged heating in distilled water (120° C, 30 min.) also had a somewhat destructive action on glucose, although much less so than heating in a phosphate solution. In the glucose solutions used by me, I observed no changes as a result of the autoclaving (reducing value, optical rotation, caramelization).

The glucose preparation employed was "Sacch. amylaceum puriss. anhydr. DAB 6 Schering—Kahlbaum". 10 ml of the glucose solutions, ten-fold concentrated in respect to the final concentration desired, were added to the flasks containing 90 ml of the nutrient solution (concentrated so as to give the right concentration after addition of the glucose).

Cellulose-wadding was chosen as the source of cellulose in Experiments 1—2, while in Exp. 3 below cotton-wool was used (cf. p. 30). At intervals a number of flasks were taken out and analyzed. The determinations of residual cellulose were made as reported above on p. 19. The glucose determinations were made according to the method of BERTRAND (see BARMANN and MYRBÄCK 1941 p. 1062) in Exps. 1—2 or that of SOMOGYI (1937) in Exp. 3 (cf. p. 65). For the determinations the culture filtrates were used, after deproteinization. For this purpose the method suggested by FOLIN and WU (1919) with Na_2WO_4 and H_2SO_4 or (in Exp. 3) the method devised by SOMOGYI (1930) with ZnSO_4 and NaOH ¹ were used. It was established by adding known amounts of glucose to culture filtrates and subsequent precipitation and sugar determination that the glucose added was recovered completely when using these precipitation methods.

¹ The "macro technique, first procedure" was used. 1 ml of each reagent was added to 10 ml of filtrate.

2. Experiments.

Experiment 1.

In this the effect of increasing amounts of glucose on growth and cellulose decomposition was studied. 4—6 parallel flasks were taken out after 10, 20 and 30 days respectively, analyses being made of residual cellulose, and in the filtrates, of pH and glucose. Table 7 gives the figures obtained. In the accompanying curves (Figs. 3—5) the course of cellulose and glucose decomposition is more clearly seen.

Fig. 3 shows the decomposition of cellulose on addition of increasing amounts of glucose; Fig. 4 shows the decomposition of "total substance". The values for "total substance" are calculated as the sum of decomposed cellulose and decomposed glucose, the latter divided by two. This was done because it was apparent that glucose was used at a rate about double that of cellulose. To create a basis for direct comparison the values for glucose consumption have been divided by two and the sum entered in the Tables and Figures ($c + g/2$). In Fig. 5, finally, the values for cellulose decomposition are plotted against the corresponding values for glucose decomposition.

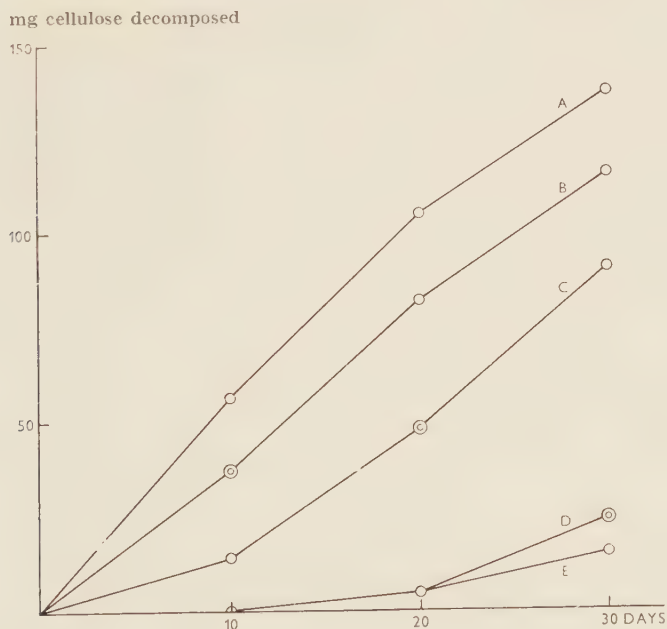


Fig. 3. Decomposition of cellulose by *Cytophaga* 3. 0, 50, 100, 250 and 500 mg glucose added in A, B, C, D, and E respectively.

Table 7. Cellulose and Glucose Decomposition in Cultures of *Cytophaga* 3.

Original Values: cellulose 503 mg, pH 7.3. Temperature: 25° C.

Glucose added mg/flask	0		50		100		250		500	
<i>10 days:</i>										
Cellulose and glucose recovered mg/flask	cell.	cell.	gluc.	cell.	gluc.	cell.	gluc.	cell.	gluc.	
	438	464		486	27	501	179	504	451	
	445	468		486	27	506	185	502	455	
	449	464		491	29	504	195	501	496	
	448	466		488	32	503	250	503	503	
	454	466		494	47					
Mean values.....	447	466	0	489	32	504	202	503	476	
Cellulose and glucose consumed.....	56	37	50	14	68	0	48	0	24	
c + g/2	56	62		48		24		12		
pH (mean)	7.1	7.0		7.0		7.1		7.1		
<i>20 days:</i>										
Cellulose and glucose recovered mg/flask	399	420		437		492	25	500	307	
	396	420		451		499	25	496	297	
	398	419		447		497	49	498	292	
	397	421		461		509	107	497	337	
	402	420		479				499	328	
	424							496	354	
Mean values.....	398	421	0	455	0	499	51	498	319	
Cellulose and glucose consumed.....	105	82	50	48	100	4	199	5	181	
c + g/2	105	107		98		104		96		
pH (mean)	6.9	6.8		6.8		6.5		6.6		
<i>30 days:</i>										
Cellulose and glucose recovered mg/flask	361	386		402		472		483	224	
	363	379		397		483		493	232	
	367	378		414		475		487	268	
	367	383		435		486		486	249	
	407							491	264	
Mean values.....	365	387	0	412	0	479	0	488	247	
Cellulose and glucose consumed.....	138	116	50	91	100	24	250	15	253	
c + g/2	138	141		141		148		142		
pH (mean)	6.8	6.7		6.6		6.3		6.2		

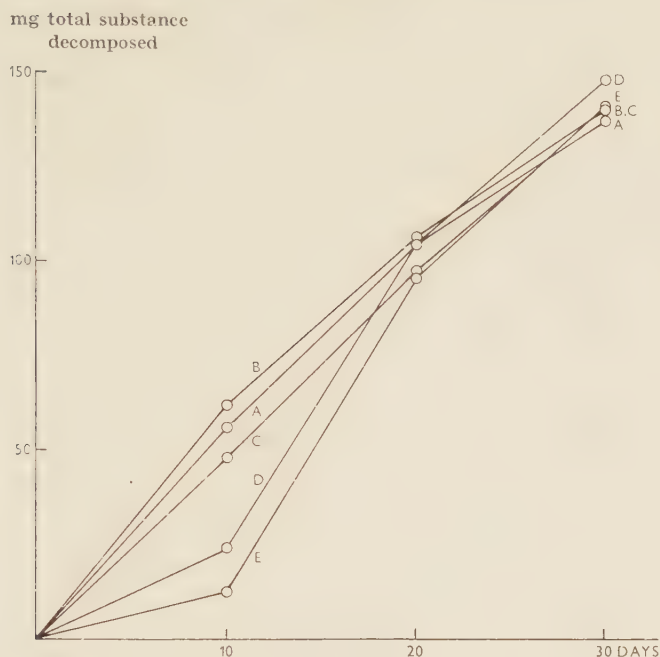


Fig. 4. Decomposition of cellulose and glucose (total substance) by *Cytophaga* 3. Designations as in Fig. 3 above.

From the Table values and the curves based thereon the following conclusions can be drawn.

1. In all cases the glucose is preferentially used as a source of carbon. The cellulose is not attacked until most of the glucose has been consumed.
2. In consequence of this the cellulose decomposition is retarded in flasks containing glucose. This retardation is proportional to the amount of glucose added.
3. When the glucose is completely used up, indicated by a double ring on the curves, the cellulose decomposition proceeds at the same rate as in flasks without any addition of glucose. For this reason the "total substance decomposed" ($c + g/2$) is fairly constant in all series.
4. On addition of large amounts of glucose (0.25 and 0.5 %) one observes the usual inhibitory effect, resulting in very low values for glucose consumption at the first sampling (10 days). The inhibition once eliminated, the rate of decomposition is as high as at lower glucose concentrations.

From the curves in Fig. 4, the rate would appear to be even higher in flasks D—E than in A—C. This is, however, somewhat misleading.

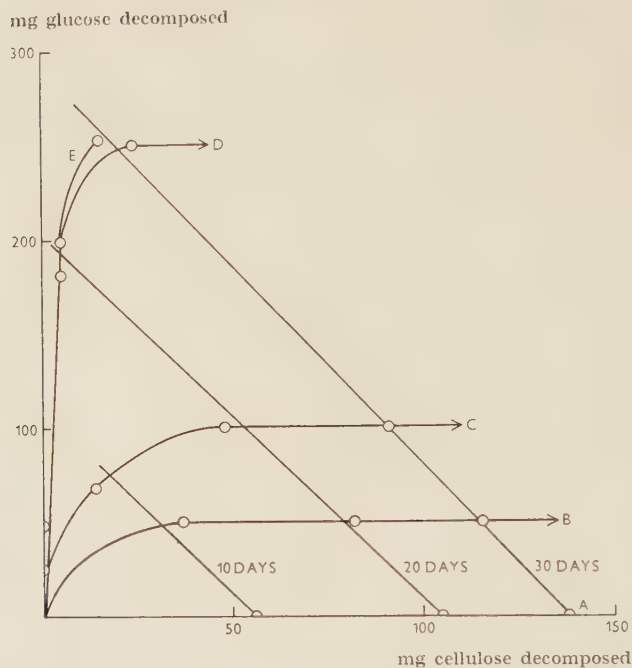


Fig. 5. Decomposition of cellulose and glucose by *Cytophaga 3*. Values for cellulose decomposition on the X axis, those for glucose decomposition on the Y axis. Designations as in Fig. 3 above.

Curves A—C do not in reality give quite a correct picture of the course of decomposition, since no determinations were made during the first rapid stage of development (from the 4th day onward). If 3 or 4 days were taken as starting-point, all curves would run about parallel. In spite of the initial inhibition curves D—E reach at last the same terminal point as do A—C. This may be explained by the fact that the growth in flasks A—C is inhibited in its turn by metabolic products formed.

That the proportion between the rates of glucose and cellulose decomposition respectively, is just 2 : 1 is possibly only a chance coincidence. I have not been able to find a plausible explanation of this fact. It may, however, be noted that H. and S. KRZEMIENIEWSKI (1937, Table on p. 46) give values which show the very same relation.

Regarding the pH values it will be seen that there exists a certain difference between cultures with and without glucose respectively, the utilization of glucose resulting in lower pH values. This is probably connected with the formation of a certain amount of acid, which is greater in the presence of glucose (cf. FÅHRÆUS 1941 p. 267).

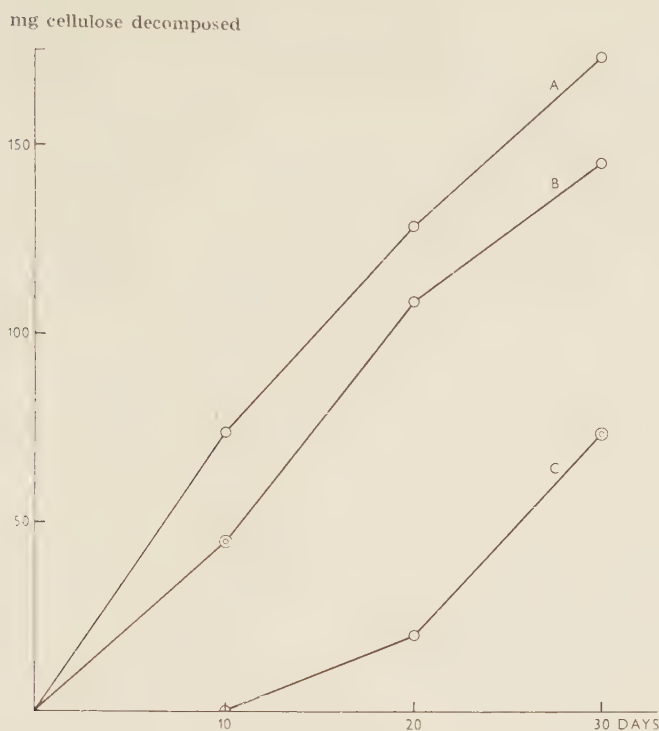


Fig. 6. Decomposition of cellulose by *Cytophaga globulosa*. 0, 50 and 100 mg glucose added in A, B, and C respectively.

Experiment 2.

The results reported in the pages above were obtained with the strain *Cytophaga 3*, newly isolated from soil. In order to examine whether the results had a wider bearing, another *Cytophaga* strain was selected for comparison, namely *C. globulosa*, which was obtained from Professor STAPP (cf. p. 13).

In a preliminary experiment with tubes containing various concentrations of glucose the limit concentrations were tested. It was found that in my experiments *C. globulosa* had a somewhat lower sensitivity to glucose than in the experiments reported by STAPP and BORTELS (1934). It was, on the other hand, more sensitive than *Cytophaga 3*, as mentioned above. A 0.1 per cent glucose medium permitted a slow development, although a marked retarding effect was observed. It must, however, be pointed out that I used a mineral medium different from that of STAPP and BORTELS, which may have affected the results.

Table 8. Cellulose and Glucose Decomposition in Cultures of *Cytophaga globulosa*.

Original Values: cellulose 502 mg, pH 6.9. Temperature 25° C.

Glucose added mg/flask	0	50	100
<i>10 days:</i>	cell.	cell. gluc.	cell. gluc.
Cellulose and glucose recovered mg/flask.....	418	460	No visible attack.
	430	458	No determinations made.
	424	451	
	436	466	
	434	450	
Mean values.....	428	457 0	
Cellulose and glucose consumed.....	74	45 50	
c + g/2	74	70	
pH (mean)	6.8	6.6	
<i>20 days:</i>			
Cellulose and glucose recovered mg/flask.....	368	400	467
	372	386	471
	366	391	480
	374	395	491
	383	393	502
Mean values.....	373	393 0	482 27
Cellulose and glucose consumed.....	129	109 50	20 73
c + g/2	129	134	57
pH (mean)	6.7	6.5	6.6
<i>30 days:</i>			
Cellulose and glucose recovered mg/flask.....	319	354	416
	321	365	432
	318	349	419
	331	351	426
	351	363	445
Mean values.....	328	356 0	428 0
Cellulose and glucose consumed.....	174	146 50	74 100
c + g/2	174	171	124
pH (mean)	6.5	6.4	6.1

A quantitative experiment was then set up, the same arrangements being used as in the preceding experiment with *Cytophaga* 3. The results will be seen from Table 8 and Fig. 6.

The values obtained indicate that there is substantially no difference between *Cytophaga* 3 and *C. globulosa*, as far as the utilization of glucose is concerned. The difference lies in a somewhat greater sensitivity to higher glucose concentrations in *C. globulosa*. It is evident that even 100 mg of glucose in each flask, i. e. 0.1 %, initially inhibit the development completely. The inhibition once eliminated, the development proceeds in the normal way.

From qualitative trials carried out later, it is evident that *Cytophaga globulosa* also is able to utilize cellobiose and mannose as sources of carbon. The inhibitory action of these sugars was less pronounced than that of glucose.

Experiment 3.

In order to obtain more complete information about the effect of glucose on the cellulose decomposition by *Cytophaga* a third experiment was set up. In this the effect of adding glucose to vigorously growing cultures of *Cytophaga* was studied. A number of flasks with Waksman's medium, containing cotton-wool but no glucose, were inoculated concurrently with *Cytophaga* 3 and *C. globulosa*. After 10 days' incubation 250 and 100 mg of glucose respectively were added to certain flasks, while other flasks were withdrawn for analysis. The remaining flasks were analyzed after 14, 20, and 28 days.

The values obtained in this experiment will be seen in Table 9 and Figs. 7 and 8.

The results obtained in the last experiment agree on the whole with those in the first ones. The inhibition caused by the glucose is now less apparent, probably owing to the much greater amount of bacteria present. In Experiments 1 and 2 above some 500,000 cells were introduced into each flask as inoculum, whereas in Experiment 3 more than 10^9 cells were present at the addition of glucose (whether all these were in fact living, cannot be decided). Such a quantity is certainly more resistant to the adverse effect of the glucose than a smaller one.

Owing to the sensitive method of sugar determination used in the last experiment very small amounts of sugar were detectable. It will be seen that also in the series without any addition of glucose a certain quantity of reducing substance occurred. This is perhaps not necessarily glucose, although the calculation is made as if this were the case. The formation of this reducing substance is dealt with in greater detail below, see p. 66.

Table 9. Cellulose and Glucose Decomposition in Cultures of *Cytophaga* 3 and *C. globulosa*.

Original Values: cellulose 481 mg, pH 6.9. Temperature 30° C.

Strain	Cytophaga 3		Cytophaga globulosa	
Glucose added mg/flask	0	250	0	100
<i>10 days:</i>				
Cellulose recovered mg/flask	cell.	gluc.	cell.	gluc.
	392		385	
	394		389	
	396		396	
	389		393	
	389		387	
	384		389	
Mean values	391		390	
Cellulose consumed	90		91	
pH (mean)	6.7		6.7	
<i>14 days:</i>				
Cellulose and glucose recovered mg flask	338	375 189	352	362 25.9
	352	391 187	341	365 29.5
	352	392 183	334	366 28.3
	352	387 176	334	358 25.8
	347	394 175	335	365 26.9
	341	388 167	335	364 30.0
Mean values	347 0.6	388 180	339 0.4	363 27.7
Cellulose and glucose consumed	134	93 70	142	118 72
c + g/2	134	128	142	154
pH (mean)	6.6	6.5	6.6	6.4
<i>20 days:</i>				
Cellulose and glucose recovered mg/flask	292	389 123	272	318 2.3
	295	393 134	295	339 2.4
	292	394 125	277	335 2.1
	308	384 141		322 2.2
		388 135		331 2.1
Mean values	297 1.4	390 132	282 1.0	329 2.2
Cellulose and glucose consumed	184	91 118	199	152 98
c + g/2	184	150	199	201
pH (mean)	6.6	6.4	6.6	6.3

(Cont.)

Strain	Cytophaga 3		Cytophaga globulosa	
Glucose added mg/flask	0	250	0	100
28 days:	cell. gluc.	cell. gluc.	cell. gluc.	cell. gluc.
Cellulose and glucose recovered mg/flask	238	384 77	207	273
	239	390 67	222	276
	247	384 78		258
	237	393 35		275
				268
Mean values	240 1.8	388 64	215 1.6	270 2.7
Cellulose and glucose consumed	241	93 186	266	211 97
c + g; 2.....	241	186	266	260
pH (mean).....	6.5	6.2	6.4	6.3

From the results obtained it is strikingly obvious that glucose is preferentially used as a source of carbon. When 250 mg of glucose are added to the flasks with *Cytophaga 3*, the decomposition of cellulose is stopped immediately. This is not due to some inhibitory effect of the glucose, for the glucose is now attacked instead of the cellulose. That the decomposition of cellulose is interrupted so suddenly is, however, rather remarkable. Glucose is certainly more readily utilized, but one would expect that the cellulose-decomposing enzyme system would act to a certain extent independently of the added glucose. It might perhaps be reasonable to assume that there exists an equilibrium: cellulose $\rightleftharpoons$ glucose, which is disturbed by the addition of glucose, but this assumption is contradicted by enzyme experiments in the presence of still greater amounts of glucose (see p. 96); in these no complete arrestation of the hydrolysis process took place, although a certain reduction in the rate of hydrolysis was noted.

The checking of the decomposition process is more probably connected with the absence of any extracellular cellulase in the medium, which could carry on the cellulose decomposition process (cf. p. 74). When glucose enters the cells, no cellulase is required as long as sufficient amounts of glucose are available. The cellulase is perhaps not formed again until the concentration of glucose sinks below a certain level, which seems to lie at about 0.1 per cent.

In the case of *Cytophaga globulosa*, where only 100 mg of glucose were added no complete arrestation of the cellulose decomposition occurred. As in Experiment 2 the decomposition is, however, retarded to an extent corres-

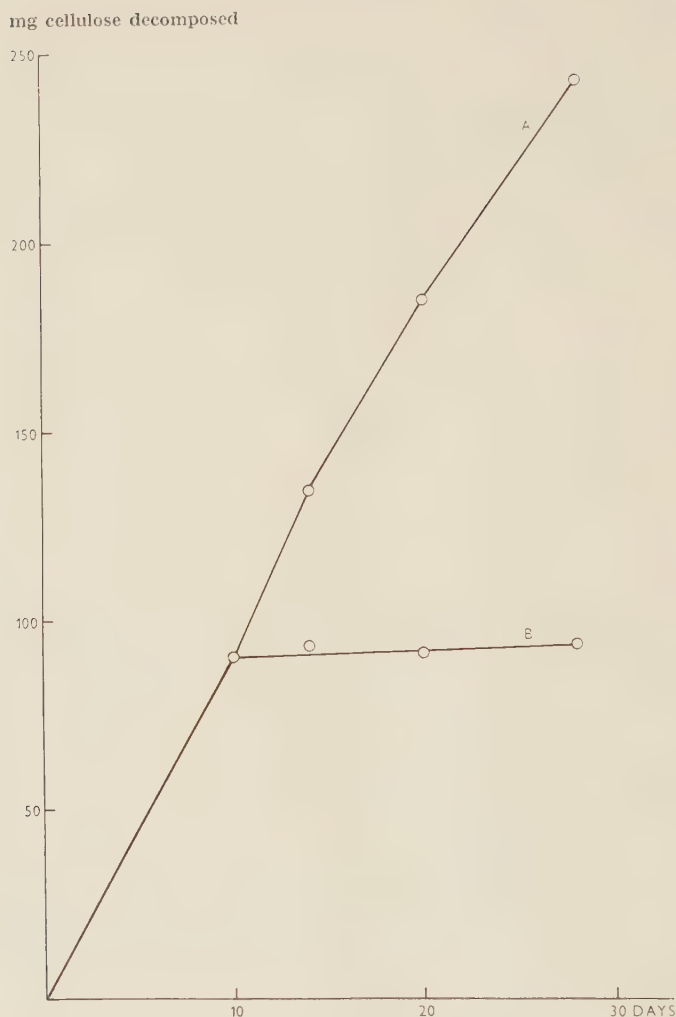


Fig. 7. Decomposition of cellulose by *Cytophaga 3*. 0 and 250 mg glucose added in A and B respectively.

ponding to the amount of glucose consumed. The earlier found constancy of the value $c + g/2$ is apparent also here. For *Cytophaga 3*, the values $c + g/2$ do not agree at the 20 and 28 days' sampling. This is possibly due to the low pH value reached, which reduces the rate of glucose decomposition.

From the above experiments it might be considered fully established that glucose is in fact a good source of carbon for the strains investigated. Because of the homogeneity from a physiological point of view that characterizes the *Cytophaga* species, it seemed very probable that this applied also to other forms described in the literature. Regarding the strains investi-



Fig. 8. Decomposition of cellulose by *Cytophaga globulosa*. 0 and 100 mg glucose added in A and B respectively.

gated by STANIER (1942 a, b), HARMSSEN (1946) and others, this is now confirmed. The negative results of all other investigators must in my opinion be explained by the fact that no accurate chemical determinations have been made. The consumption of the small amounts of glucose present in the cultures has therefore escaped their observation entirely. The inhibitory influence of even small quantities of glucose has erroneously been taken as a proof that the glucose is not utilized as a nutrient substance.

D. Other Sugars.

Judging from the controversial results obtained with glucose, the suitability of other sugars should also be subjected to renewed examination, since in these cases no quantitative experiments have been carried out which clearly demonstrate the opposite fact. As mentioned above, cellobiose and mannose are used by both strains studied by me; cellobiose is utilized also by all strains examined by STANIER (1942 b), mannose and in addition xylose are utilized by his strain *Cytophaga rubra*, but not by *C. Hutchinsonii* and *Sporocytophaga myxococcoides*. The sugars named are all sterically very nearly related.

Regarding other sugars it seems, however, probable that they are not able to support growth of *Cytophaga* (the name used in the older, restricted sense, cf. p. 11). In any case the sugars in question are not preferred to cellulose. This is apparent from Table 5 above. In the corresponding experiment *Cytophaga* 3 decomposed cellulose vigorously in media containing as much as 1.0 per cent of all sugars in question except fructose. At this concentration a growth at the expense of the respective sugar must have been clearly observed. The sugar was in these cases consequently not preferentially utilized. Decomposition to a small degree of the sugars added cannot, however, be excluded without quantitative experiments. Future investigations will probably throw more light upon these matters.

It should be mentioned here that in enzyme experiments reported later (p. 117), cellobiose was the sole disaccharide which was hydrolyzed by *Cytophaga* preparations.

Fructose, which is very susceptible to degradation during the heat sterilization, should also be further examined before any sure conclusions are drawn.

E. The Inhibitory Effect of Glucose.

The problem of the inhibitive action of glucose must now be discussed in greater detail. First it may be pointed out that this effect is especially obvious when a small or normal inoculum is used (less than 1 million cells, roughly estimated). It seems that the adverse effect is partly overcome if a greater quantity of bacteria is present (cf. p. 47). Moreover, *Cytophaga* can apparently be adapted to greater amounts of glucose through repeated subculturing on this sugar, and an adapted strain will grow well on glucose even after transferring a smaller number of bacteria. See in this connection Fig. 6 in an earlier paper (FÄHRÆUS 1941).

The above-mentioned phenomena are normally met with in microorganisms, although the inhibitory agents may be of other kinds.

The strongly inhibitory influence of glucose on *Cytophaga* in usual experiments remains, however, to be accounted for. One might think of osmotic conditions (BOKOR 1930, p. 23), but that seems highly improbable considering the very small concentrations necessary for complete inhibition. In addition, the osmotic effect would also be observed in the case of other sugars. Another explanation would be a lowering of the redox-potential through the reducing properties of glucose, but IMŠENECKI (1941), who considered this possibility, came to the conclusion that this could not be the case. STANIER (1942 a, b) observed in connection with a Warburg experiment with unsterilized glucose that autoclaved glucose solutions exhibited a striking toxic influence on *Cytophaga* compared with glucose not heated but filtered through a Seitz filter (cf. p. 40). He concluded that the glucose itself was not toxic but that toxic substances were formed on heat sterilization.

This discovery enabled STANIER to cultivate his *Cytophaga* strains in tubes devoid of cellulose but with glucose in concentrations up to 2 per cent, without any inhibition being observed. If, on the other hand, the glucose was sterilized together with the nutrient medium, no growth occurred in concentrations above 0.1 %.

It is not clear whether STANIER has also tested glucose which has been autoclaved separately and not together with the nutrient medium. There is, in fact, a great difference between these two methods, as already mentioned above on p. 40. In my experiments I have always sterilized the glucose solutions separately and added them aseptically to the experiment tubes in order to avoid a destruction of the glucose. However, the glucose has always had the unfavourable influence so often referred to in about the same concentrations as STANIER and others have found.¹

Some further points also deserve consideration. The source of nitrogen used by STANIER in the experiments mentioned is not clear. The use of tap water for the medium implies that some unknown factors may have influenced the results.

When the work of STANIER became available to me, I at once tried the filtration method for obtaining sterile glucose solutions. But in spite of the non-heating of the glucose solutions, substantially the same results were obtained. Experiments with *Cytophaga globulosa* showed clearly that 0.1 % solutions of glucose markedly retarded growth, 0.25 % of the filtered glucose no longer permitting any growth of *Cytophaga globulosa*.

¹ In some recent experiments irregularities have occurred which I am not able to explain. In these experiments *Cytophaga globulosa* grew readily on 0.5 % autoclaved glucose.

The discrepant results arrived at by STANIER and the present author might perhaps be explained by assuming that the glucose preparations themselves contained inhibitive substances and that the sterilization only to some extent increased the unfavourable effect.

In all earlier experiments the preparation "Sacch. amyl. puriss. anhydr. DAB 6" of Schering—Kahlbaum had been used. Consequently some other preparations available (Merck puriss. DAB 6, American Standard Samples no. 41) were tried. The results, however, were still the same as earlier. An attempt at purification through treatment with animal charcoal was also unsuccessful. Growth was obtained in a 0.1 % solution but not in a 0.5 %, in spite of the fact that some glucose had been adsorbed by the charcoal and consequently the concentration was somewhat less than the figures given indicate.

A recrystallization of the glucose was then tried. Glucose was dissolved in water to saturation. To the solution the tenfold amount of absolute alcohol was added and the solution was inoculated with some minute crystals of glucose and allowed to stand for some days in a refrigerator. The glucose crystallized was sucked off and washed with some ice-cooled absolute alcohol, dried *in vacuo* and used for an experiment. By way of comparison untreated glucose was tested. Both were sterilized by filtration through a Seitz filter.

The results were, however, entirely negative. Growth was obtained in tubes with cellulose + 0.1 % glucose (both recrystallized and original glucose preparations) but not in tubes with 0.25 % glucose.

Since the available chemical preparations of glucose had proved unsuitable, I thought it would be possible to obtain glucose free from the inhibiting substances through the action of the bacteria themselves. An enzyme experiment was therefore set up, according to the methods described later on p. 84, in order to get sufficient amounts of glucose through enzymic hydrolysis of lichenin. The purification of the sugar solution obtained was carried out in accordance with the description on p. 72 (sugar from cellophane). The use of temperatures higher than 40° C was avoided. The purified glucose was used for a new experiment in tubes with nutrient medium, containing 0.05, 0.1, 0.2 and 0.4 % glucose respectively. The tubes were inoculated with *Cytophaga globulosa*. The result was, however, still completely negative, growth being observed in the two lowest concentrations, but not in 0.2 and 0.4 %. No inhibitory substances were derived from the lichenin, since this substance, as proved in controls with lichenin instead of glucose, permitted growth even in a 0.4 % concentration.

The experiments related above do not argue in favour of the idea that the inhibition of *Cytophaga* by glucose media is connected with some toxic impurities in the glucose formed at autoclaving, although the results of STANIER point to this being the case. The present author regards it as an alternative possibility that the glucose itself exhibits in some way or other the inhibitory action towards *Cytophaga*. Such an inhibitory effect

would not be unique, since certain enzyme systems are inhibited by glucose (for references see GALE 1943).

Thus the discrepancy between STANIER's and my own results is so far unexplained. That a real difference should exist between our *Cytophaga* strains seems to be less probable.

Finally, some words should be said about the statements of various authors to the effect that certain other cellulose-decomposing bacteria are likewise incapable of utilizing glucose as a source of carbon, but are specialized on cellulose. To these bacteria belong, among others, some anaerobic cellulose-decomposers, *Clostridium cellulosae dissolvens* (KHOUVINE 1923), *Cl. cellulosam fermentans* (WERNER 1926) and *Cl. cellulosolvens* (COWLES and RETTGER 1931).

The pure culture of the thermophilic strain of ENEBO (1944) also is apparently specialized on cellulose alone. *Pseudomonas erythra*, an aerobic species described by FULLER and NORMAN (1943 b, p. 276), is supposed to be specific. One of WINOGRADSKY's (1929) *Cellvibrio* species, *C. ochracea*, was said to be specialized on cellulose.

Although it cannot be entirely excluded that such a specialization can really exist, most of the cases mentioned above should be taken under renewed consideration. The evidence brought forward in studies with *Cytophaga* indicates that similar conditions may exist also in other cellulose-decomposing organisms. At the time when several of the last-mentioned bacteria were studied, the conditions in *Cytophaga* were not yet really understood.

V. The Course of Cellulose Decomposition by *Cytophaga*.

A. The Products of Cellulose Decomposition.

The ultimate products of bacterial cellulose decomposition will be of very different kinds, owing to the great variety of bacteria engaged in this process. The following substances, among others, have been found in cultures of cellulose-decomposing bacteria: carbon dioxide; volatile acids, such as formic, acetic, propionic, butyric acid; non-volatile acids, such as lactic acid and other oxyacids; ethanol and hydrogen. Methane, which was earlier considered a typical product of certain anaerobic cellulose-decomposers, is more doubtful, since later investigators (IMŠENECKI 1940, MEYER 1943) have demonstrated the absence of this substance in pure cultures of anaerobic cellulose-decomposing bacteria.

In addition to the products named various synthetic compounds, such as pigments, different polysaccharides, etc., are also formed.

The final products in cultures of *Cytophaga* are — apart from the cell material — mainly carbon dioxide (BOKOR 1930, IMŠENECKI and SOLNCEVA 1936, WALKER and WARREN 1938, STANIER 1942 b) and the “mucilage” mentioned earlier (p. 13). Other products come far behind: viz. small amounts of some volatile acid (HUTCHINSON and CLAYTON 1919, FÄHRÆUS 1941), a non-volatile acid (WALKER and WARREN 1938), an alcohol-soluble pigment, and a flavour substance. The mucilage is dealt with in detail below. The production of volatile acids has been doubted by many investigators (WINOGRADSKY 1929, IMŠENECKI and SOLNCEVA 1936, JENSEN 1940, STANIER 1942 b) and deserves more careful examination. I have carried out some experiments in order to study this problem further, but since it lies outside the main scope of the present work, these experiments will be recorded elsewhere.

The intermediary products, however, are just as important as the final products in the breakdown of cellulose, for in a heterogeneous population, as it exists under natural conditions, the intermediate substances can be intercepted by the action of other microorganisms and thus escape further attack by the real cellulose-decomposers. They will then serve as material for the formation of widely different end-products.

In searching for intermediary substances the building units of cellulose: cellobiose and glucose, suggest themselves. One would imagine that the hydrolysis of the cellulose to these compounds is the simplest way of breaking down the polysaccharide. This has, in fact, been demonstrated in a number of cases.

PRINGSHEIM (1912) was the first who clearly showed the formation of the above-mentioned simpler sugars in cultures of bacteria growing on cellulose.¹ He studied cultures of anaerobic and thermophilic cellulose-decomposing bacteria and succeeded in checking the growth ("Sistierung", "sisting") without the activity of the hydrolyzing enzymes being arrested. This could be achieved through the addition of toluene or other antiseptics to vigorously fermenting cultures. By changing the temperature from 55° C to 20° C in cultures of thermophilic bacteria the same result was obtained. PRINGSHEIM was in that way able to bring about an accumulation of reducing sugars, which could be identified as cellobiose and glucose.

With similar methods it has been shown that the cellulose is hydrolyzed to cellobiose and glucose by several cellulose-decomposing bacteria and fungi. For references compare FAHRÆUS (1944 p. 2); some additional publications will be mentioned below.

POCHON (1935, 1943) has with *Plectridium cellulolyticum* obtained a large yield of glucose through adding xylene to strongly fermenting cultures or through arresting the bacterial metabolism by lowering the pH some units. With a second strain isolated, *Terminosporus thermocellulolyticus*, the same result was obtained with the former method, the alteration of pH, on the other hand, remaining without effect (POCHON 1942, 1943).

HUNGATE (1944) has proved the formation of cellobiose, but no glucose, by the anaerobic mesophilic species *Clostridium cellobioparus*.

As regards fungi some important work has been carried out concerning the cellulose-splitting enzyme system. This work will be resumed in connection with my own enzyme experiments, see p. 79.

Very little is known of the subsequent splitting of glucose by the cellulose-decomposing organisms. It has presumably been assumed for the most part that the above-mentioned metabolic products (acetic acid etc.) have arisen through fermentation of glucose. In cases in which the cellulose-decomposer has also been grown on glucose medium (SIMOLA 1931 b, IMŠENECKÍ 1940) the same products appear to have been obtained as in cultivation on cellulose. In this connection SIMOLA's report of the occurrence of acetaldehyde in cultures of cellulose-decomposers is of interest. Recently, NORD and SCIARINI (1946) have reported the formation of alcohol by *Merulius niveus* and *M. tremellosus* growing on cellulose and other carbohydrates under anaerobic conditions. In the presence of air, the alcohol seems to be completely dehydrogenated, and no intermediates are to be found.

While a simple hydrolytic course of decomposition thus seems clearly established in a number of cases, the conditions in *Cytophaga* are less obvious by far. Of all aerobic cellulose-decomposers these have been considered the most specialized on cellulose, and since it was known that glucose

¹ Some analogous experiments were made with fungi as early as in 1903 by VAN ITERSON.

was unsuitable as a nutrient substance one could not well think of a hydrolytic course of degradation. HUTCHINSON and CLAYTON (1919) found small amounts of a volatile acid, and in addition a bacterial mucilage, supposed to be of a pectic nature, in the cultures of "*Spirochaeta cytophaga*", but no reducing substances. They expressed no opinion on the course of cellulose decomposition.

Then WINOGRADSKY (1929) put forward the idea of oxydative decomposition, a suggestion that has been made also in the case of several cellulose-decomposing fungi (MARCUSSEN 1926, BOSWELL 1941). This theory later received considerable attention and was accepted by various investigators. Consequently it will be discussed here in more detail.

B. Theory of Oxydative Decomposition. The Mucilage.

This theory was founded on two facts: the complete absence of reducing sugars in the culture liquid of *Cytophaga*, and the extensive production of the mucilage which possessed properties similar to those of "oxycellulose".

WINOGRADSKY (1929) found that the mucilage exhibited acidic properties, was coloured by methylene blue and other basic dyes and was soluble in alkaline solutions, from which it could be precipitated with acid. These properties were in good accordance with those of chemically produced oxycellulose (the acidic type). Considering at the same time the total absence of reducing properties in the mucilage and culture liquid of *Cytophaga*, he came to the conclusion that the cellulose was oxidized at the 6th carbon atom of the glucose unit and possibly also at other carbonyl groups in the molecule, in this way yielding the oxycellulose-like mucilage.

It may be noted that WINOGRADSKY believed in an oxydative breakdown also by the *Cellvibrio* species which he had studied. In this case, however, the only reason for his opinion seems to have been some colour reactions. No mucilage is formed by *Cellvibrio*.

WINOGRADSKY himself made no quantitative chemical analyses of the mucilage in *Cytophaga*, but later LOICJANSKAJA (1937) and WALKER and WARREN (1938) have carried out such analyses.

They were able to show that the mucilage contained uronic acid units and was consequently of a polyglucuronide nature. In this respect it agreed with chemically produced oxycellulose. Accordingly these authors came to the same conclusion as WINOGRADSKY regarding the origin of the mucilage.

BOSWELL (1941) strongly supported this theory. He suggested that "in certain cases the decomposition of cellulose is facilitated by oxidation resulting in the formation of oxycellulose containing uronic acid molecules". After this loosening of the molecule the cellulose was supposed to be degraded to low molecular products. What products would be derived from oxycellulose in this process is not clear. As regards

certain anaerobic bacteria, BOSWELL suggested that the polyglucuronide formed might possibly be decarboxylated and hydrolyzed to pentose, as the bacteria in question were capable of fermenting pentoses, but not hexoses. This, however, cannot be assumed to happen in cultures of *Cytophaga* species, which are in general not able to utilize pentoses.

Upon closer examination one finds that the basis of this "oxycellulose theory" is rather weak, as pointed out by several authors, for instance already by SIMOLA (1931 b p. 23). The idea that the mucilage in *Cytophaga* is not an oxidation product of cellulose but rather a synthetic product of the microbial cell was later advanced by IMŠENECKI and SOLNCEVA (1936), who found that the mucilage retained acidic dyes equally as well as basic ones.

NORMAN and BARTHOLOMEW (1940) undertook an investigation into the mucilage of some cellulose-decomposing organisms, among others two *Cytophaga* strains.

They concluded that the mucilage is a bacterial gum and not a kind of oxycellulose for mainly the following reasons: a) The mucilage is closely associated with the bacterial cells. b) Any oxydative process would give rise not only to short-chain soluble fragments but also to long-chain insoluble material which should be easily detectable. On analysis it was, however, found that the residual cellulose was not more oxidized than initially. c) The presence of pentose units in an oxycellulose from cotton is impossible, but the furfural yield in the determinations exceeded that which would be expected on the basis of uronic units present, and implies that some pentose groups must be involved. d) Oxycellulose could only be formed by an extra-cellular oxidative process, which would be unique, if the cell was intact, and no energy would be derived by the cell until chain-splitting had taken place to such an extent that the fragments were water-soluble and therefore could pass into the cell.

It is true that some of the above reasons are open to criticism¹, but on the whole they seem to be sufficient for rejecting WINOGRADSKY's theory of oxydative decomposition. In the publication cited no opinion was expressed about the real course of decomposition in the cases investigated. Later NORMAN and FULLER (1942 p. 248) put forward the opinion that the process was most probably a hydrolytic one.

In this connection a recent work by WAGNER, WEBBER and SIU (1947) is worth mentioning. The authors point out that certain oxidized cellulose derivatives, such as 6-carboxy-cellulose and cellulose-2,3-dialdehyde are more resistant to attack of the fungus *Metarrhizium glutinosum* than the cellulose itself.

NORMAN and BARTHOLOMEW (1940) were able to confirm the occurrence of uronic groups in the mucilage formed by various *Cytophaga* strains.

¹ The authors do not accept an oxydative decomposition because intermediates are not to be found; but no hydrolysis products had been established either.

Moreover, they found polysaccharides containing uronic acid in all of seven cellulose-decomposing strains investigated (not *Cytophaga* alone). This lends further support to the fact that polyuronides are indeed very wide-spread in the capsular substances of various bacteria (for references see FÄHRÆUS 1944 p. 14).

It is of importance to observe that many of these bacteria are incapable of utilizing cellulose as a source of carbon. Consequently, these bacteria build up the cell polysaccharides containing uronic acid from simpler carbohydrates, for instance glucose or even less complex compounds. The fact that products containing uronic acid are formed by cellulose-decomposing organisms is consequently no proof that they arise through a direct oxidation of cellulose.

There is, however, an easy way of determining whether the mucilage arises through direct oxidation of cellulose or whether it is a product synthesized from low molecular substances. By culturing *Cytophaga* concurrently on cellulose and glucose substrates, and by comparing the mucilages formed, one should be able to get an answer to this question.

This has been done by STANIER (1942 b) and myself. STANIER states that "liquid glucose cultures of *Cytophaga Hutchinsonii* may become so viscous that they are hard to pipette. The material can be precipitated from such cultures by alcohol or acetone, and exhibits all the properties which were reported by WINOGRADSKY and by WALKER and WARREN as characterizing oxycellulose. Were the material really an oxycellulose, it should of course be produced only as a result of growth on cellulose" (p. 177).

I have myself made an experiment in order to compare the mucilage formed on cellulose and that formed on glucose (also reported in my earlier paper, 1944). Because of the inhibitory influence of higher glucose concentrations on *Cytophaga globulosa* this offered some difficulties, but it was found that this strain could be adapted to larger amounts of glucose by repeated subculturing on increasing concentrations. In this way it was possible to cultivate it on a 0.25 % concentration of glucose, without cellulose. When most of this glucose was consumed, which was proved by analysis, aseptically new glucose was added so as to reach a 0.25 % concentration again.

The cultures were arranged as previously indicated (p. 17) but on a larger scale. One-litre Erlenmeyer flasks with 300 ml of the usual mineral medium and 3 g cotton-wool or 0.75 g glucose respectively (the glucose being aseptically added to the autoclaved medium), were inoculated with a suspension of *Cytophaga globulosa*. For inoculation of the glucose flasks a culture was chosen which was adapted to glucose as mentioned above. At the end of about 14 days at 30°C the experiment was terminated. By

examination of microscopic slides and by inoculation on plain agar with glucose, it was verified in the case of each flask separately that no infection had taken place. In the preparation of the mucilage the method of WALKER and WARREN (1938) was followed on the whole, but it was found that the addition of alcohol was not necessary, the mucilage flocculating out completely already by addition of acid. After precipitation the liquid was centrifuged, the mucilage washed on the centrifuge with alcohol and ether, and dried *in vacuo*.

In these operations the mucilage obtained from cellulose cultures and that from glucose cultures showed no differences. The yield of substance, on the other hand, was somewhat larger when culturing *Cytophaga* on glucose (cf. Table 4, FÄHRÆUS 1944).

That bacterial mucilage is formed to a greater extent in culturing on glucose than on cellulose is a fact that can always be observed when cultivating these organisms. This fact may possibly be explained as a response to unfavourable conditions, as shown in the case of other bacteria (HOOGERHEIDE 1939).

As the amount of substance thus obtained was limited, the greater part of the crude products was used directly without previous purification, for uronic acid determination. The uronic acid was determined by distilling with 12 % HCl according to TOLLENS and LEFÈVRE's method, as modified by NANJI and collaborators (1925), i. e. the method used by WALKER and WARREN when examining the mucilage of *Cytophaga*.¹ Instead of standard baryta solution ascarite was used, however, as absorbent for the carbon dioxide, and this gave more reliable results. The use of ascarite offered a further advantage, namely that samples could be taken out every hour by two absorption vessels being used alternately. In this way the exact course of CO₂ evolution could be registered. By this means, as WHISTLER and collaborators (1940) showed, it is possible to decide whether there is in the sample a pure uronic acid, a carbohydrate free from uronic acid or a mixture of both. This is shown by the shape of the curves (see WHISTLER, l. c.).

The values obtained after 4 hours' distillation are given in Table 10.

Owing to the very limited amount of mucilage available for the determinations, too great importance must not be attached to the absolute values. However, there seems to be an essential difference in the amount of

¹ It has been pointed out by MATTSON and KOUTLER-ANDERSSON (1944) that not only uronic acids give off CO₂ when boiled with 12 % HCl. The main scope of the investigation reported here was, however, to establish the analogous constitution of the mucilage in cellulose and glucose cultures respectively. The results do not seem to be invalidated by the findings of MATTSON.

Table 10. Uronic Acid Content of the Mucilage of *Cytophaga globulosa*.¹

	Amount analyzed g	CO ₂ evolved mg	% CO ₂	Difference (see p. 62)	% of uronic acid anhydride	
Mucilage from glucose cultures {	0.5053	3.6	0.71	0.74	0.47	1.9
	0.3109	2.4	0.77			
Glucose (2 det.).....	5.000	13.5	0.27			
Mucilage from cellulose cultures {	0.2794	2.2	0.79	0.89	0.62	2.5
	0.2247	2.2	0.98			
Cellulose (2 det.).....	2.500	5.5	0.22			

carbon dioxide evolved between, on the one hand, cellulose and glucose and, on the other, the bacterial substance obtained on these substrates. This difference is clearer still, if one examines the curves that it has been possible to draw on the basis of the time course of the carbon dioxide evolution (Fig. 9).

The differences pointed out by WHISTLER and collaborators (l. c.) between glucose and products containing uronic acid, as regards the shape of the curves, are clearly shown here. Judging from the shape of the curve the cotton cellulose used might also contain traces of uronic acid.

The real uronic acid content of the mucilage has been calculated by subtraction of the values for glucose from the values for the mucilage (after 4 hours' distillation). It is in this way that the values in the last two columns of Table 10 were obtained. This seems fully justified also as regards the polysaccharide formed in cellulose medium, as this product is built up from glucose rather than from cellulose (see below).

Thus uronic acid occurs in a rather modest amount in the crude products analyzed. The amount roughly corresponds to that found by LOIC-JANSKAJA (1937), who obtained 2 % uronic acid anhydride by direct analysis of a culture on cellulose. On the other hand, the quantity is very much smaller than the amount given by WALKER and WARREN (1938), namely 14 %, found on analysis of a purified preparation. Even if one reckons with the mucilage, when purified, gaining in uronic acid concentration, it may, however, be suspected that in this case part of the uronic acid found is derived from the medium, as cellulose-wadding seems to have been used as substrate. An analysis of the very purest Swedish cellulose-wadding

¹ Some of the figures given in an earlier work (FÄHRÆUS 1944, Tab. 5) were not quite correct, unfortunately. The correct values are given here.

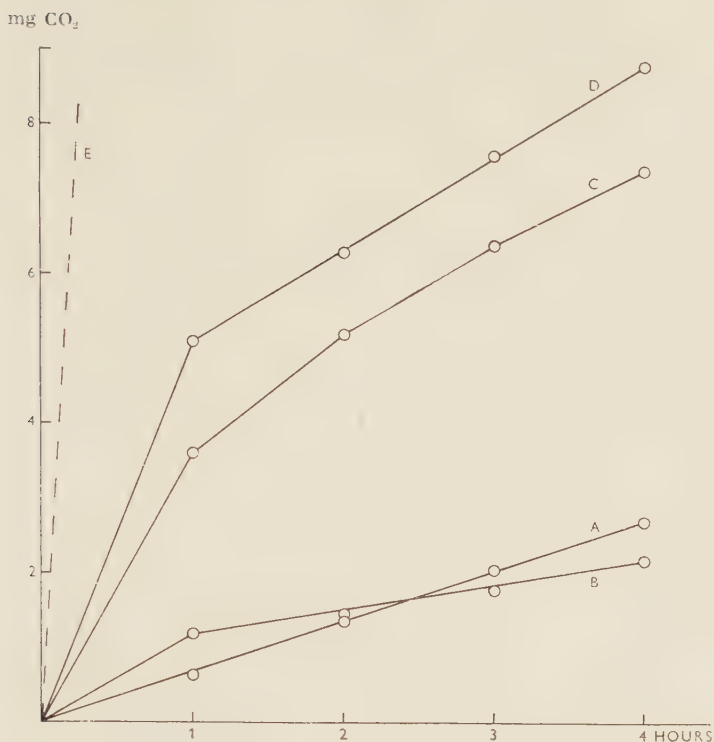


Fig. 9. Evolution of carbon dioxide by distilling with 12 % HCl from 1 g of: A) Glucose, B) Cotton cellulose, C) Mucilage from glucose culture, D) Mucilage from cellulose culture, E) Pectin. Each curve represents the mean of two parallel series of values.

has, in fact, given a content of uronic acid almost double that given by cotton-wool.

As a result of the comparison of the mucilage from cellulose and from glucose it may be said that as regards the uronic acid content no essential difference appears to exist. The mucilage formed in glucose cultures contains uronic acid as well as that formed in cultures with cellulose, and might therefore equally well be called "oxycellulose". That the mucilage formed in glucose cultures should be an oxycellulose seems absurd, however. Because of the agreement mentioned between this mucilage and that formed in cultures on cellulose there is no reason either in the latter case to assume that the mucilage occurs through direct oxidation of cellulose. On the contrary, it should be regarded as a purely synthetic product.

The opinion of NORMAN and collaborators (1940, 1942), STANIER (1942 b) and others has thus been largely confirmed by my results. There no longer exists, then, any other reason for assuming an oxydative attack on the

cellulose than the indirect one, namely that glucose has never been found as an intermediate product in the cellulose decomposition by *Cytophaga*.

The possibility of a hydrolytic decomposition will now be considered in greater detail.

C. Theory of Hydrolytic Decomposition.

The "sisting method" devised by PRINGSHEIM (1912) to demonstrate reducing substances in cultures of cellulose-decomposing bacteria was mentioned earlier (p. 57). No attempt was, however, made by HUTCHINSON and CLAYTON (1919) or by WINOGRADSKY (1929) to accumulate sugar by this method. BUCKSTEEG (1936) later made such an experiment but was unsuccessful. He added toluene to cultures of *Cytophaga* which showed vigorous development. Periodically samples were withdrawn and tested on reducing substances. All determinations were, however, negative.

Later investigations have, on the whole, also failed to prove the occurrence of any reducing substances in *Cytophaga* cultures, even if different artificial means were applied in order to accumulate reducing sugar (JENSEN 1940, STANIER 1942 b).

IMŠENECKI (1938) is the only one who has reported the formation of reducing substances in *Cytophaga* cultures, although traces only were found. When sealing cultures of aerobic cellulose-decomposers with paraffin according to the method of KALNINŠ (1930), he was able to prove traces of reducing substances in cultures of *Mycrococcus* (= *Cytophaga*) *Hutchinsonii*, while at the same time obtaining large amounts of such substances in cultures of *Cellvibrio vulgaris*. He made, however, no attempt to isolate and identify the reducing substance. Because of the minute quantities formed this was probably not possible. He drew, however, the conclusion that the substance formed was glucose and that the cellulose was hydrolyzed to glucose.

As mentioned above, JENSEN (1940), using the same method, was nevertheless unable to establish any reducing substance in *Cytophaga* cultures, but like IMŠENECKI was able to do so in *Cellvibrio* cultures.

In view of my above-related results showing that glucose was in fact a suitable source of carbon for *Cytophaga*, no obstacle seemed to exist for assuming a hydrolytic course of decomposition. But as long as no hydrolytic products had really been proved, this would have to remain merely an assumption. However, I thought that, in some way or other, it might be possible to show the formation of hydrolytic decomposition products also in *Cytophaga* cultures.

First some experiments were carried out which followed up earlier works on this subject (PRINGSHEIM 1912, KALNINŠ 1930, SIMOLA 1931 b, BUCKSTEEG 1936).

1. "Sisting" experiments.

Experiment 1.

Cultures of *Cytophaga 3* and *Cytophaga globulosa* with strong bacterial development (14 days old) were treated in different ways in order to arrest the bacterial growth without stopping the hydrolyzing enzymes. The treatment was carried out in accordance with the methods of PRINGSHEIM (1912) and KALNIŅŠ (1930). The following series were set up:

1. Controls. Untreated.
2. Flasks sealed with paraffin.
3. Flasks with addition of toluene (new-distilled).
4. Flasks with temperature raised from 30° to 40° C.

In the experiment the usual 300 ml flasks were employed with cotton-wool in Waksman's medium (see p. 17). The flasks were inoculated concurrently with *Cytophaga globulosa* and *Cytophaga 3*. After incubation for a period of 14 days at 30° C, 5 flasks were taken out for immediate cellulose and glucose determinations and the remaining flasks were treated in the ways indicated above. In the toluene series 5 ml of toluene were added to the 100 ml of nutrient medium. The toluene had to be renewed during the course of the experiment.

Careful control was kept to make sure that none of the analyzed flasks were infected, which might possibly have upset the results. This control was carried out by examining microscopic slides and by transferring a loopful from each flask to plain agar. The determination of reducing sugar was carried out in the filtrates after deproteinization with ZnSO_4 and NaOH , according to SOMOGYI's method (1930, cf. p. 40). For the determination of reducing sugar the semi-micro method of SOMOGYI (1937) was chosen, as it was to be expected that very small quantities of sugars would appear in the cultures. The great sensitivity of the SOMOGYI method has been attained by saturation of the reagent solution with sodium sulphate, which reduces the solubility of the oxygen and thereby the reoxidation of precipitated Cu_2O . It was impossible to procure such a high concentration of sodium sulphate as that indicated by SOMOGYI, however; the reagent used by me contained only 125 g Na_2SO_4 per litre instead of 200 g.

With the reagent used by me sugar values were obtained that agreed very well, amounting to only 0.5 mg per 100 ml, i.e. 0.0005 %. A standard curve for the reagent was plotted on the basis of values obtained by using known amounts of glucose.

The values obtained in the analysis of cellulose, reducing substance and pH are shown in Table 11.

It will be seen from the Table that even the controls show a slight but distinct accumulation of reducing substances. The cellulose decomposition has proceeded here with a normal course and considerable rapidity. In

Table 11. Cellulose Decomposition and Formation of Reducing Substance in Cultures of *Cytophaga* 3 and *C. globulosa*.

The Reducing Substance Calculated as Glucose. Original Values: cellulose 473 mg, reducing substance no trace, pH 7.2. Temperature 30° C.

Treatment	Controls		Toluene added	Flasks sealed	Temp. raised
	14 days	28 days	28 days	28 days	28 days
	<i>cell. gluc.</i>	<i>cell. gluc.</i>	<i>cell. gluc.</i>	<i>cell. gluc.</i>	<i>cell. gluc.</i>
<i>Cytophaga</i> 3.					
Cellulose recovered and glucose formed mg/flask	338 0.9	217 2.3	326 1.0	¹ 330 0.4	228 2.8
	343 0.9	220 2.2	321 1.1	264 1.6	237 3.2
	344 1.0	223 2.5	330 1.0	252 1.5	¹ 243 2.0
	339 1.1	223 2.4	331 1.1	222 2.2	226 2.9
	327 1.1	210 2.5	330 1.0	232 1.7	223 3.2
Mean values.....	338 1.0	219 2.4	328 1.0	243 1.8	229 3.0
Cellulose consumed.....	135	254	145	230	244
pH (mean)	6.7	6.6	6.8	6.5	6.6
<i>Cytophaga globulosa</i> .					
Cellulose recovered and glucose formed mg/flask	336 0.5	200 2.2	333 1.0	219 1.7	214 4.1
	322 0.6	198 2.2	336 1.1	206 2.1	215 4.2
	333 0.6	205 2.2	330 1.3	209 1.8	233 3.8
	339 0.5	198 2.1	334 1.1	239 1.7	222 3.7
	339 0.5	198 2.4	329 1.2	228 1.6	¹ 296 1.8
Mean values.....	334 0.5	200 2.2	332 1.1	220 1.8	221 4.0
Cellulose consumed.....	139	273	141	253	252
pH (mean)	6.7	6.5	6.7	6.5	6.5

¹ Flasks infected by moulds or bacteria. Not included in calculation of mean values.

the flasks with toluene, on the other hand, the cellulose decomposition has been entirely arrested and reducing substances occur in quantities as small as before the addition of toluene. This result shows clearly that the conditions are different from those known for *Cellvibrio* (KALNIŅŠ 1930, JENSEN 1940), for instance, and implies perhaps a quite different manner of enzyme action in *Cytophaga*. These matters will be discussed later.

In the flasks sealed with paraffin only relatively small amounts of reducing substances appeared (less than in the controls). The cellulose decomposition is also somewhat retarded as compared with the controls.

Since the access of air was restricted, this seems quite reasonable. Concerning this series it should be observed, however, that the volume of air in the flasks was fairly great, which naturally diminished the effect, if any, of the sealing.

Only in the experiment with a rise in temperature has a somewhat greater increase in the concentration of reducing substance occurred. The cellulose decomposition is not arrested, although it is a little weaker than in the control flasks. One might, therefore, imagine that the optimum for the appearance of the reducing substances possibly lies at an even higher temperature. In order to examine this possibility a new experiment was set up.

Experiment 2.

The arrangement was the same as in the preceding experiment. Thus the flasks were kept 14 days at 30° C, after which some flasks were left as controls and the rest were placed in different incubators with temperatures ranging from 35° to 65° C. In order to reduce evaporation of water at the higher temperatures the cotton plugs had been covered with rubber cloth. In any case sterile water had to be added once in order to keep the volume unchanged. After an additional 14 days the experiment was terminated and the usual analyses were made.

The experiment was carried out exclusively with *Cytophaga globulosa*. The results will be seen from Table 12. The values given (mg/flask) are mean values of 4 parallels, which showed good agreement.

As seen from the Table, the same effect of the rise in temperature was obtained as in the previous experiment, although here it was less distinct perhaps. An optimum seems to lie at 40° C, and a rise in temperature above

Table 12. Cellulose Decomposition and Formation of Reducing Substance in Cultures of *Cytophaga globulosa*.

The Reducing Substance Calculated as Glucose. Original Values: cellulose 487 mg, reducing substance no trace, pH 6.9.

Temperature °C	30	30	35	40	45	50	55	60	65
Days	14	28	28	28	28	28	28	28	28
Cellulose consumed....	151	275	284	277	161	155	154	153	152
Glucose formed		1.7	2.1	2.7	1.2	1.0	0.9	0.9	0.9
pH (mean)	6.6	6.7	6.7	6.6	6.8	6.8	6.7	6.6	6.5

this point has not resulted in larger amounts of reducing substances. The growth of the bacteria and the decomposition of the cellulose apparently reach a maximum at about the same temperature, above which the decomposition has entirely ceased. By transferring from these flasks to tubes with the usual nutrient medium it could be shown that the bacteria had not survived the temperatures above 40°C .

The result of certain experiments carried out later (p. 105), where bacteria preparations were extracted with alkaline solutions at pH 9, suggested that it might be possible to extract the cellulose-decomposing enzymes from the cells suspended in the culture liquid. An adjustment of the pH to 9 for a short period in order to get the cellulase into solution seemed to be a possible way. For this purpose the following experiment was set up.

Experiment 3.

As in the preceding experiments the flasks were kept 14 days at 30°C , after which period a number of flasks were left as controls. The remaining flasks were treated as follows.

NaOH was added until pH 9.0 was reached, and the flasks were left so overnight in a refrigerator. Then the liquid was neutralized with HCl and the flasks incubated at 30° or 40°C . Toluene was added to some of the flasks after the neutralization, the others being given no toluene. Thus, the following series were started:

1. Controls. 30°C .
2. Controls. 40°C .
3. Treated at pH 9. 30°C .
4. As in 3. Toluene added.
5. Treated at pH 9. 40°C .
6. As in 5. Toluene added.

The addition of NaOH and HCl was made aseptically with sterile solutions.

After 14 and 28 days analyses were made as in the preceding experiments. No positive result was, however, achieved with the different treatments carried out. Thus it seems to be of little use to list the figures obtained in the analyses. A somewhat increased formation of reducing substance had taken place in the flasks at 40°C , as in the earlier experiments. The treatment at pH 9 only had the effect of retarding the growth for a shorter or longer period; in general the cultures treated in this way had, however, recovered and showed almost as extensive a cellulose decomposition as

those without this treatment. Toluene had, as usual, inhibited the further development totally.

Later two other ways were also tried in order to accumulate reducing sugars in the *Cytophaga* cultures. In one experiment pH was lowered to 6.0 by addition of acid after a period of 14 days (cf. POCHON's experiment, cited on p. 57), and in a second experiment NaF (0.2 per cent)¹ was added after the same period. In no case, however, was a positive result obtained.

All the above experiments may be said on the whole to confirm earlier experiences of the formation of reducing substances in *Cytophaga* cultures. It is true that a formation of that kind has undoubtedly taken place, but the amounts obtained are very small and cannot be proved with the macromethods generally used. By means of the usual Bertrand method for determining reducing sugar I had myself no positive evidence of such a formation.

In any case it is not established that the reducing substance formed is really cellobiose or glucose; it might well be a cellulose dextrin with lower reducing power. The substance is formed also in normal cultures, and it is difficult to understand why glucose should be formed in this case but should be almost totally consumed when added in somewhat greater quantities (see experiments on pp. 41—51). A small amount of glucose, formed by hydrolysis, might, however, be adsorbed to the mucilage and escape further attack. The higher values obtained at 40° C might then be explained by the fact that more mucilage is formed at this temperature, which increases the adsorbing surface. The higher amount of reducing substance formed on addition of glucose in Exp. 3, Tab. 9, should also be observed. The glucose increases the quantity of mucilage formed (cf. p. 61).

One might also think of some kind of autolysis products being gradually formed in the cultures. Such products, however, would very probably also, and perhaps to an even greater degree, be formed by checking the bacterial development through addition of antiseptics.

Through the "sisting" experiments the problem regarding the course of cellulose decomposition was not solved. Yet there was another way of attacking the problem, which had not earlier been tried with *Cytophaga* cultures. If investigations are made using bacteria preparations or enzyme solutions instead of cultures of living bacteria, it is easier to disclose the effect of the separate enzymes (cf. KARSTRÖM 1941 p. 1259).

2. Enzyme experiments.

This mode of procedure has earlier been employed by SIMOLA (1931 b) on cellulose-decomposing bacteria. He then used the spore-forming *Cellulo-*

¹ This concentration inhibits the growth of *Cytophaga*, as shown in some experiments.

bacillus myxogenes. The enzyme preparation was obtained by culturing this strain in Erlenmeyer flasks with nutrient solution and filter paper. After one week's incubation at 37° C the contents were centrifuged, and the paper mass washed and pressed to remove the water, and finally chopped into small pieces. These were used as enzyme material. The paper mass could also be extracted in water after the addition of toluene, whereupon the solution was filtered through a Berkefeld filter. A cellulase was then obtained in cell-free solution. Only a few experiments with these preparations are reported, however.

The conditions in *Cytophaga* are somewhat different. When cultured in flasks most of the bacteria are suspended in the medium. Because of the abundant slime formation it is very difficult to centrifuge the bacteria with good results. The residual cellulose contains only a very small part of the bacteria. Recently I have succeeded in getting effective bacteria preparations from such culture media, but at the beginning of the experiments this method did not seem promising.

Agar substrates were instead tried for the purpose of obtaining bacteria preparations. But the use of agar substrates for *Cytophaga* also offered very great difficulties. The reason for this lies at least partly in the sensitivity of *Cytophaga* to certain inhibiting compounds. The presence of such substances in usual filter paper has been reported earlier. Such substances are also present in the commercial agar and are apparently very difficult to wash out. Similar results were obtained by FRIES (1941) when he studied the germination of spores of certain fungi.

By a prolonged treatment with water it is possible to free the agar from the inhibitory substances to such an extent that the agar can be used in a 1.5 % concentration without inhibiting the growth of *Cytophaga*. The purification of the agar was accomplished by the method indicated by STAPP and RUSCHMANN (1924). After a previous extraction in often changed water the strips were dissolved in boiling water and amounts of 50 ml were poured into large dishes, where they were left under running tap water, and finally distilled water. On the moist surface there was formed a bacterial film, which could be scraped off. This was continued for a week. The purified agar was then collected and the substrate desired was made up.

As sources of cellulose precipitated cellulose (SCALES 1916), ground filter paper suspended in the agar, and entire paper rounds covering the surface of the agar were tried. The last method proved the best, since *Cytophaga* in any case grows only on the surface and since on agar with precipitated cellulose it forms no obvious colonies which could be easily collected. On the papers *Cytophaga* forms yellow spots of bacterial mucilage, where the fibres are entirely decomposed. It was found that a strongly attacked paper with numerous such spots could be lifted off, the mucilage adhering to the agar surface. Thus the mucilage with the embedded bacterial cells could be finally scraped off from the agar without any greater quantities of cellulose being present.

Cellophane sheets also were tried for this purpose, but without success. The importance of a carefully selected cellophane is evident from experiments carried out later, compare p. 111.

The best result was obtained if agar plates covered with filter paper were heavily inoculated with a large piece of decomposed paper, which was drawn in curves over the surface of the paper by means of a platinum needle. Then a vigorous growth was obtained along the entire inoculated line. Transfers with a smaller quantity or with a bacterial suspension only caused the appearance of numerous small points of attack but no entire line. After 2—3 weeks' incubation at 30° C the unattacked cellulose was lifted off and the bacterial mucilage adhering to the agar surface collected by means of a spatula. The cellulose in these mucilage preparations only constituted a very small part.

With the bacteria preparations obtained in this way preliminary experiments were made in order to examine whether reducing substances were formed under conditions which were considered optimal for the hydrolysis of cellulose. Easily split cellulose sources, lichenin and regenerated cellulose, were used as substrates. Details regarding these substrates are given on pp. 82 and 111.

It was in fact evident in the very first trials that in this case reducing substances were formed to a considerable extent. The work, then, was directed to finding out the optimal conditions for the hydrolysis process and, finally, to performing the hydrolysis on a scale large enough to allow a purification and identification of the reducing substance. This became possible when, ultimately, bacteria preparations could be obtained from liquid cultures, thus making larger amounts available. Regarding the methods here used, see p. 80 below.

It was in this way proved in experiments with *Cytophaga globulosa* that the reducing substance formed in the enzymic decomposition of lichenin was in reality glucose. By using regenerated cellulose as substrate the rate of hydrolysis is considerably reduced, and only small amounts of reducing substance arise. By means of concentrated enzyme solutions, which have recently been produced, the purification of the reducing substance arising through hydrolysis of cellophane has also been successful. In this case, too, it was possible to show that the substance formed was glucose.

Regarding details in the first experiments the reader is referred to the report given in an earlier work (FÄHRÆUS 1944). Only the essentials necessary for the elucidation of the problem in question, the course of cellulose decomposition, are considered here. Later (pp. 78 et sqq.)

continued enzyme experiments are related, and a more detailed report of the methods used is given there.

In order to obtain larger amounts of the reducing substance six Erlenmeyer flasks, each containing 25 ml phosphate buffer of pH 7.4 and 80 mg lichenin together with 12.6 mg finely ground bacteria preparation of *Cytophaga globulosa*, were shaken with 5 ml toluene in a water bath at 30° C for 24 hours. After that the solutions were collected and sugar determination was made. 1.9 mg/ml were obtained, if the reducing substance was calculated as glucose. The solution was then evaporated under reduced pressure (35° C) to c. 20 ml. The slightly coloured solution was then shaken with animal charcoal and filtered. On the clear, colourless filtrate polarimetric determination was made in a 2 dm tube, + 1.27° of rotation being registered. Sugar determination of the same solution gave 12.1 mg/ml, calculated as glucose. From this was obtained

$$[\alpha]_{\text{D}}^{20} = \frac{+ 1.27 \times 100}{1.21 \times 2} = + 52.5^{\circ}$$

The value obtained thus agrees with the value of the optical rotation of glucose, taken from the literature.

For additional control the phenylosazone of the reducing substance was prepared. The remainder of the filtrate after shaking with charcoal was therefore evaporated to dryness *in vacuo*, the residue being extracted with boiling methyl alcohol and filtered. After evaporation of the alcohol the residue was dissolved in water. In this solution the osazone was precipitated with phenylhydrazine hydrochloride according to VAN DER HAAR'S (1920) directions. The recrystallized osazone, whose crystal structure entirely corresponded with that of the glucosazone, had a melting point of 210° C (uncorr.), which value agrees well with that given by VAN DER HAAR. The osazone concurrently prepared from pure glucose had a melting point of 208° C.

A similar experiment was carried out with cellophane as a substrate. In this case flasks containing 20 ml phosphate buffer of pH 7.0 with MgCl₂, 1,000 mg cellophane in strips, toluene, and in addition 4 ml of a strong enzyme solution from *Cytophaga globulosa* (see p. 103) were used. The flasks were shaken in a thermostat bath at 36° C. After 48 hours the contents were collected, the residual cellophane was removed by filtration, and the solution was evaporated to dryness *in vacuo*. The remainder was extracted in hot methyl alcohol and filtered. The methanol was then evaporated *in vacuo* and the remainder was dissolved in distilled water. The slightly brown-coloured solution was shaken with charcoal (norit S. X. 30) and filtered. After this treatment determinations were

made of reducing value and optical rotation. The treatment with charcoal was repeated twice; after the second treatment $+ 0.47^\circ$ rotation was registered in a 0.445 % solution (2 dm tube), which gives a specific rotation of $[\alpha]_D^{20} = + 52.8^\circ$.

By these results it may be regarded as fully established that the reducing substance which arose out of the lichenin and the cellophane in the above reported experiments, is identical with glucose. Whether glucose was exclusively formed cannot be considered as proved, however. A small quantity of cellobiose, for instance, may possibly have arisen, but have been lost during the purification of the glucose. Since the preparations used contained a powerful cellobiase (cf. p. 117), all cellobiose that may have been formed has certainly been hydrolyzed to glucose, however.

At first it seems somewhat astonishing that glucose is formed so readily in these enzyme experiments but not in cultures of living bacteria on addition of toluene. There are, however, a number of differences in the arrangement of the experiments that may condition the difference in the results. It seems probable that the positive result in the enzyme experiments is mainly due to the use of easily split cellulose forms, regenerated cellulose and lichenin, while in the sisting experiments cotton cellulose, which is attacked with much greater difficulty, was employed.

3. Discussion.

In the enzyme experiments, it has been established that *Cytophaga globulosa* possesses the enzymes necessary for hydrolysis of cellulose (incl. lichenin) to glucose. This may be said to support the theory of hydrolytic decomposition as a normal course of cellulose breakdown in this strain. Nevertheless, such a course cannot be considered as definitely *proved*. It must be admitted that the enzymes, especially the cellulase, obtained from the bacteria are extremely weak considering the vigorous cellulose decomposition in cultures of living bacteria. An explanation would be that some essential factor, or complex of factors, is lost during the preparation of the enzyme material, but the possibility should not be overlooked that the main pathway of decomposition might be a different one.

By analogy with the enzymic breakdown of other polysaccharides (CORI *et al.* 1938), it might be supposed that the cellulose is transformed by phosphorytic decomposition. This was suggested by STANIER (1942 b) but without any experimental support. In the last part of the present paper (p. 90) some experiments are related, in which this possibility has been examined. As yet no positive evidence has been adduced to show that a

phosphorolysis would take place. But the experiments are too few for a definite opinion. So far, only experiments with lichenin have been carried out. In addition, the phosphorylase or some essential cofactors may have got lost during the preparation of the dry bacteria. The possibility of a phosphorolytic breakdown must be left open for the present. Future investigations will perhaps throw more light upon this problem.

The hydrolytic course of decomposition is so far the only course really established. But in that case why have all attempts to prove reducing substances in the cultures been unsuccessful? What is the reason for the indisputable difference between *Cytophaga* and most other cellulose-decomposing bacteria, in the cultures of which reducing substances have been frequently demonstrated?

The negative result of the "sisting" experiments seems to imply that the cellulase is not excreted into the surrounding medium, as in the case of many other cellulose-decomposing bacteria, and that the reaction between enzyme and cellulose takes place exclusively at the cell surface or inside the cell.

The mode of action of a *Cytophaga* cellulase can at present only be the subject of mere hypotheses. The matter has to some extent been treated by STANIER (1942 b), but a few words should be said here also regarding this problem. First the constitution of cellulose must be briefly considered. (For references, see for instance OTT 1943, HEUSER 1944.)

Cellulose is chemically a polysaccharide almost entirely built up by cellobiose units linked together in long chains by means of primary valences. In most natural materials the cellulose is intimately connected with other polysaccharides, lignin, etc., but in cotton fibres a fairly pure cellulose is to be found. For this reason the cotton fibre is very convenient for biochemical research purposes. The following review will be confined to this material.

The chain molecules have a considerable length, which, however, may vary within wide limits. The results obtained with different determination methods do not agree entirely. STAUDINGER and FEUERSTEIN (1936) found a degree of polymerization of c. 2,000, while GRALÉN and SVEDBERG (1943) found values up to c. 10,000. The latter value corresponds to a molecular weight of almost two millions or a chain length of 5 μ .

Of great importance is the micellar arrangement of the molecules. It has been shown that the fibre consists of crystalline parts, crystallites or micelles, in which the chains are strictly orientated. Secondary valence forces seem to be active within the micelles. The crystalline regions are, however, interrupted by areas of amorphous character. The old idea of the micelles as individual units regularly arranged in an intermicellar substance (NÄGELI) has now been abandoned, and a reticular system such as for instance that proposed by FREY-WYSSLING (1939), seems to be more able to account for the properties of the fibre. In such a system the micelles are connected with each other through an irregular network of amorphous cellulose. In addition, there seems to exist a continuous system of intermicellar spaces of varying size. These

may contain water and other substances. The size of the micelles seems to average $60 \times 600 \text{ \AA}$. The long chain molecules are thus much longer and they will pass through numerous micelles.

The existence of the micelles and the long chain molecules explains the great tensile strength of the cellulose. The micellar arrangement is probably also the cause of the considerable enzyme resistance of native cellulose. FAUST, KARRER and SCHUBERT (1928) pointed out that rayon produced under stretching of the fibre, which promotes micellar orientation, has an increased resistance to the *Helix* cellulase (cf. p. 110).

The micelles are arranged in a system of superior order, the fibrillar system, which can be made visible microscopically by various treatments. The fibrils are spirally wound around the axis of the cotton fibre at an angle of about 30° (cf. Pl. 2).

The constitution of lichenin and regenerated cellulose (cellophane) is treated later (pp. 84 and 111).

The observation has been made by all investigators that the *Cytophaga* cells become regularly oriented parallel with the fibrillar structure of the fibre, consequently at a certain angle to the fibre axis (see also Pl. 2). The ability of orientation in such a manner is probably connected with the particular morphology and mode of locomotion in *Cytophaga* (cf. p. 11). Through their elasticotactic behaviour (STANIER 1942 b, c) the vegetative *Cytophaga* cells move parallel with the fibrils and occupy a suitable position in very intimate contact with the fibre surface. The creation of a large contact surface is apparently an important condition for an effective attack on the cellulose, and is perhaps facilitated by the great flexibility of the vegetative cell. MEYER (1943) suggested that the cellulose-decomposing anaerobe studied by him might possess short plasma protuberances, which could be introduced into the hollows of the fibre. WINOGRADSKY (1929) once suggested that there existed some sort of "haustoria" in *Cytophaga* which might serve as locomotion organs. If such haustoria really existed, they might conceivably be able to enter the intermicellar spaces of the fibrils. In this way a most effective attack on the amorphous regions would be brought about. In any case it seems probable that the amorphous cellulose is first attacked. In this way the micelles would be gradually isolated. A decomposition of the micelles might perhaps be facilitated by a partial incorporation in the cell after dissolution of the surrounding amorphous cellulose. (The long chain molecules have about the same length as the *Cytophaga* cells (cf. above), but the length and thickness of the micelles may only amount to c. $1/50$ of that of a *Cytophaga* cell.)

There were reasons for thinking that the cellulase of *Cytophaga* would be extractible. This was the background to Experiment 3 above (p. 68). That this experiment remained unsuccessful may indicate that the enzyme quantity present is too small. Possibly a continuous consumption and reformation of cellulase takes place, for which the metabolism of the living

cell is necessary. A measurable hydrolysis, then, cannot be demonstrated, unless very great amounts of bacteria, as in the enzyme experiments, are present.

D. Summary and Conclusions of Parts IV and V.

From various quarters an oxydative course of cellulose decomposition by *Cytophaga* has been claimed. This theory has been based mainly on three facts:

- 1) It had been found that glucose was unsuitable as a source of carbon for *Cytophaga* and even injurious in higher concentrations;
- 2) It was to be expected that reducing sugars would arise as intermediates in a hydrolytic breakdown of cellulose, but all experiments had failed to establish even traces of such substances;
- 3) It had, on the other hand, been observed that the mucilage formed in the *Cytophaga* cultures was similar to chemically produced oxycellulose.

By some authors (IMŠENECKI and SOLNCEVA 1936, IMŠENECKI 1938, WAKSMAN 1940) a hydrolytic course of decomposition was, however, suggested, but in these cases without the support of convincing experiments. More careful experiments were later carried out by NORMAN and BARTHOLOMEW (1940), STANIER (1942 a, b) and myself (1941, 1944). Through these investigations the above reasons for assuming an oxydative decomposition have been largely invalidated.

Thus it was shown by STANIER and myself that glucose was readily utilized by all the *Cytophaga* strains examined. On the basis of the present writer's results, reported in Part IV above, the following conclusions may be drawn.

Glucose is preferentially used as a source of carbon by *Cytophaga*. Consequently, when cellulose and glucose are simultaneously present in the culture medium, the latter substance is first removed by the bacteria. The cellulose is not attacked until the glucose concentration has reached a fairly low level. The matter is to some extent obscured by the inhibitory effect of higher glucose concentrations on the growth of *Cytophaga*; the real cause of this inhibition is not quite clear. The disappearance of the glucose from the culture medium can, however, be easily established by using low concentrations of glucose, which do not exert an inhibitory influence.

The problem given in Point 2) above was more difficult to solve, since all attempts to prove the formation of glucose in living cultures of *Cytophaga* remained without success. The question could, however, be approached

in another way. By various methods it was possible to produce dry bacteria and enzyme solutions of *Cytophaga*, and by means of such preparations a hydrolysis of lichenin and cellophane could be obtained. It was demonstrated that the sugar arising was glucose. In this way it was shown that the *Cytophaga* cells contain cellulose-hydrolyzing enzymes and that, consequently, a hydrolytic course of decomposition is possible. The conclusion has been drawn that this is probably the normal course of cellulose decomposition in *Cytophaga*.

The glucose which is formed as an intermediate in the hydrolysis, never becomes apparent in the presence of living bacteria, as the rate of glucose consumption exceeds that of cellulose decomposition considerably (p. 41). That glucose cannot be demonstrated even after arresting the bacterial growth is probably due to some particular properties in *Cytophaga*, this question being discussed on pp. 73 et sqq.

Finally, the mucilage formed in the *Cytophaga* cultures has been examined by NORMAN and BARTHOLOMEW and also by the present author. It has become highly probable that this substance does not originate directly from the cellulose, but that it is of synthetic nature. Thus this reason for assuming an oxydative decomposition has also been removed.

It is true that only a few *Cytophaga* strains have been more closely examined from these points of view. Similar results have, however, been obtained with both microcyst-forming and non-microcyst-forming species (STANIER 1942 b). Considering the homogeneity from a physiological point of view that characterizes the cellulose-decomposing *Cytophaga* species, it appears probable to the author that the same course of decomposition applies to all species in the group.

The possibility of a phosphorolytic decomposition of cellulose by *Cytophaga*, as suggested by STANIER (1942 b) for instance, has been considered (see also p. 90), but the experiments carried out do not suffice to give a final answer to this question. No evidence has, however, been obtained which indicates a phosphorolytic breakdown.

The subsequent decomposition of the glucose formed in a hydrolytic decomposition by *Cytophaga* has not been studied. This problem presents an interesting field for future investigations. There very probably exist analogies with the course of glucose decomposition in many other bacteria.

VI. Further Investigations into the Enzymic Hydrolysis of Lichenin and Cellulose by means of *Cytophaga* Preparations.

A. Review of the Literature.

The enzymes so far known to be involved in the hydrolysis of cellulose (incl. lichenin) to glucose are cellulase, lichenase and cellobiase. Each of these may exist in several modifications, since different pH optima and other divergences are reported by various investigators. According to WEIDENHAGEN'S (1932) system, the cellobiase should be identical with β -glucosidase from almond emulsin, but although the latter enzyme has generally been thought to be capable of splitting cellobiose, it is certainly not identical with most cellobiases from microorganisms, which are frequently only slightly or not at all able to split salicin. In addition, ZECHMEISTER, TÓTH and FÜRTH (1940) have shown that a chromatographic separation of cellobiase and salicinase in almond emulsin is possible. It seems more reasonable to speak of enzyme classes, such as the β -glucosidases, α -glucosidases, etc. (GRASSMANN and associates 1933 b, PIGMAN 1944).

FREUDENBERG and PLOETZ (1939) suggested that cellulases in different organisms may contain different "carriers", but nothing is known with certainty in this respect. No authentic example of the dissociation of any carbohydrase in enzyme and carrier has in fact been given (PIGMAN 1944).

In nature the cellulases always appear to be accompanied with lichenases, since all hitherto described cellulose-decomposing enzyme mixtures from different sources are also able to hydrolyze lichenin. Earlier (PRINGSHEIM and SEIFERT 1923, KARRER *et al.* 1924, GRASSMANN *et al.* 1933) cellulase and lichenase were not clearly distinguished, but ULLMANN (1932)¹ and FREUDENBERG and PLOETZ (1939) advanced the opinion that they are two distinct enzymes. It is also obvious that there exist a number of bacteria which are capable of utilizing lichenin but not native cellulose (own experiments). This may indicate that the bacteria in question do not possess the true cellulase.

Investigations into the cellulose-decomposing enzymes have earlier been

¹ The basis for ULLMANN'S opinion is very weak, however.

carried out mainly with material from invertebrates (KARRER and collaborators 1923 et seq.¹, BOYNTON and MILLER 1927, RIPPER 1930, TRAGER 1932, MANSOUR and MANSOUR-BEK 1937, PARKIN 1940), barley malt (PRINGSHEIM and collaborators 1923, 1926, 1928) and fungi (GRASSMANN and collaborators 1931, 1933, BOSE and collaborators 1937, 1939¹, PLOETZ 1939, SAKSENA and BOSE 1944). The best known object is the common edible snail, *Helix pomatia*, the digestive juice of which contains a powerful cellulase. It has been studied most extensively in the above-mentioned important papers by KARRER and associates. PLOETZ (1940) has used the *Helix* cellulase in a series of investigations into the enzymic hydrolysis of wood.

Experiments with bacterial cellulases have been carried out only to a very limited extent. SIMOLA (1931 b) studied briefly the cellulase in *Cellulobacillus myxogenes* and the present author (1944, 1946) made some preliminary investigations into the enzymic hydrolysis of lichenin and regenerated cellulose with enzymes from *Cytophaga*.

The results of KARRER and associates obtained with the *Helix* enzymes can be summarized shortly as follows. Lichenin was completely broken down to glucose by the crude enzyme mixture, which also split starch and inulin, for instance (1924 p. 144). After dialysis of the enzyme mixture only cellulose, lichenin, cellobiose and gentiobiose were hydrolyzed (1924 p. 916). By adsorption to aluminium hydroxide the cellobiase could be removed and the lichenin was then split up to a triose, lichotriose (1925 p. 248). Rayon and cotton fibre were attacked at a much lower rate than lichenin. When using very concentrated enzyme solutions the authors succeeded in demonstrating a considerable hydrolysis even of such substrates (1925 p. 797).

Some kinetic studies were made, and it was found that, in the first stage, the hydrolysis of lichenin followed the course of a monomolecular reaction (1923). The amount of enzyme present and the quantity of substrate broken down were not proportional, the double enzyme amount splitting only a substrate quantity 1.5 times that split by the simple one in equal periods of time (1923).

The pH optimum was found to be about 5.3, with both lichenin and regenerated cellulose as substrates. The addition of various sugars inhibited the hydrolysis considerably. A concentration of 0.1 per cent glucose (that occurring through the hydrolysis not calculated) depressed the rate of hydrolysis to about $\frac{2}{3}$ of its normal value (1924 p. 916).

GRASSMANN and associates (1931, 1933 a, b) studied the enzyme mixture obtained from *Aspergillus oryzae* and other moulds (the technical preparation "luizym"). The properties of the lichenase and cellulase were the same as those reported by KARRER in most respects.

The cellobiase and cellulase could be separated from each other by adsorption to aluminium metahydroxide. It was found that the cellulase was specific for cellulose and cellulose dextrins, but split the higher oligosaccharides only slowly and cellobiose scarcely at all. The cellobiase, on the other hand, hydrolyzed the oligosaccharides from the hexaose downwards. This fact is, according to the authors, an indication

¹ Older work on cellulose-splitting enzymes should be sought here.

of the mode of action of the cellulase: it attacks central oxygen bridges but not the linkages near the end of the molecule.

In contrast to the results of KARRER, but in accordance with the results of PRINGSHEIM and BAUR (1928) on malt lichenase, the authors found a proportionality between the enzyme quantity and the substrate converted, until 40 per cent decomposition was reached. The optimum pH range was 4—5 (cellulase, lichenase and cellobiase). GRASSMANN and associates carried out their experiments — owing to the pH optimum found — in an acetate buffer, but they demonstrated that phosphates had a favourable influence on the rate of hydrolysis. (Cf. VEIBEL 1941, pp. 1770—71.)

FREUDENBERG and PLOETZ (1939) investigated the *Helix* cellulase and the luizym mixture. In both cases they could partly separate cellulase, lichenase and cellobiase from each other by fractionated alcohol-ether precipitation. The cellulase and lichenase were found to have somewhat different pH optima.

The present writer has carried on the preliminary experiments with enzymes from *Cytophaga* which were begun some years ago. Much of the work necessarily had to be devoted to cultivation and preparation methods, since the bacteria in question, like most other cellulose-decomposing bacteria, had not been studied enzymically before. The experiments are being continued, and it is hoped that future results will give a more thorough knowledge of the enzyme system of these organisms.

B. Methods.

1. Bacteria preparations.

The first bacteria preparations were obtained by using agar plates covered with filter papers, as reported above on p. 71. It is, however, evident that in this way only limited amounts of bacteria become available. A further great disadvantage is the difficulty of keeping such plates sterile, since they have to be opened several times. The amount of bacteria obtained proved sufficient, however, for the first experiments, and the formation of glucose from lichenin could be demonstrated by using such preparations.

For the production of greater quantities of bacteria a cultivation in flasks containing liquid media was desirable. Some experiments were carried out with folded filters standing in the flasks with the peak upwards. In such a way a fairly great surface with optimal moisture was obtained, and after inoculation an extensive growth took place in the surface zone. Yet great difficulties were experienced in separating the bacteria from the paper.

For this reason flasks with totally immersed cellulose were finally tried. With most *Cytophaga* strains examined by me the preparing of bacteria from such cultures offers considerable difficulties or is at least tedious, since large volumes of liquid must be concentrated *in vacuo*, if special precipitation methods cannot be applied. Such methods seemed to have a destructive effect on the enzyme system, however. The bacterial cells embedded in the mucilage could be precipitated by means of alcohol, but the enzyme system appeared to be severely damaged by the alcohol treatment. More active preparations were obtained by using ammonium sulphate as a precipitant. At 20 per cent saturation the mucilage was in general completely precipitated. Such preparations had to be dialyzed to remove the sulphate.

In the case of *Cytophaga globulosa* it was possible to take advantage of the fact that the mucilage is readily precipitated by acid without the addition of alcohol. A complete flocculation of the mucilage takes place when the pH is lowered to about 3.5.¹ This fact, so far unique, as no other *Cytophaga* strains studied by me behave like this², may be due to a different chemical composition of the mucilage or cell surface. What difference is responsible for this effect has not been investigated.

In the beginning the preparing through precipitation with acid did not seem very promising, the preparations obtained being rather inactive, but it was found later that the activity could be raised considerably if certain precautions were taken. This will be reported in greater detail on p. 102.

Bacteria preparations have also been produced by concentrating the culture liquid *in vacuo* after dialysis.³ The products obtained had a greater content of salts, since a complete dialysis was difficult to effect. The activity of such preparations was good and the yield of preparation was greater in this method than in the HCl precipitation method.

The bacteria preparations naturally contain several enzymes, some of which are not directly engaged in the decomposition of the cellulose. The enzymes here concerned are only the enzymes or the enzyme complex occupied in the hydrolysis of cellulose (incl. lichenin) to glucose. This process, also in *Cytophaga*, is probably brought about by at least two distinct enzymes, cellulase and cellobiase. The data given below apply to the whole system, since attempts to separate the enzymes were not made until

¹ Such preparations have been called H-preparations.

² With excess of HCl, as in the washing procedure described on p. 19, the mucilage of *Cytophaga 3* was also precipitated.

³ Such preparations have been called C-preparations.

quite recently and no experiments have so far been carried out with the separate enzymes.

In all enzyme experiments reported below, dry preparations of *Cytophaga globulosa* have been used, unless otherwise stated.

2. Substrates.

In the enzyme experiments mainly lichenin and cellophane have been used as substrates.

For the production of lichenin Iceland moss (*Cetraria islandica*) was collected. The preparation of the lichenin followed the old indications given by HÖNIG and SCHUBERT (1887) as modified by KARRER, JOOS and STAUB (1923), HESS and FRIESE (1927) and HESS and LAURIDSEN (1940).

The air-dried, cleansed lichens were weighed in 300 g portions, each of which was extracted with 6 litres of 2 per cent K_2CO_3 , which was changed every day for a week. The lichens were washed free from alkali by means of often changed water. The lichenin was then extracted through boiling in water for 24 hours. The hot solution was filtered through cloth in a Büchner funnel and was subsequently frozen out. After complete freezing out, and subsequent thawing, the lichenin was separated from the mother liquor. This procedure was repeated five times, the lichenin being frozen out each time in order to facilitate the filtration. Finally, the lichenin was washed with water, until no reaction with an I-KI solution (HESS and FRIESE 1927) was visible any longer. The moist lichenin was then transferred to 2 litres of 95 per cent alcohol, which was changed twice. The alcohol treatment was followed by a similar ether treatment. The excess ether was finally sucked off, and the residue was allowed to evaporate slowly, the lichenin being placed in a desiccator which was connected to a vacuum-pump and sucked empty from time to time. By the very gradual drying achieved in this way the porous structure and the good solubility of the lichenin is preserved (KARRER *et al.* 1923). For complete drying the lichenin was heated in an oven at 50° C. The dry lichenin was ground in a mill and was used for the experiments without further purification. It was relatively dark-coloured, and certain preparations had a small reducing value, as determined according to WILLSTÄTTER and SCHUDEL (see below p. 85).

The yield in respect to dry lichens was about 15 per cent. The air-dried lichenin contained about 10 per cent water and 0.4—0.5 per cent ash.

The lichenin solutions used in the experiments were always prepared immediately before the experiments were started. The phenomenon known as "aging" of the lichenin, pointed out by PRINGSHEIM and BAUR (1928) and GRASSMANN and associates (1933 a) may thus have influenced the results. However, it was evident that no constant rate of decomposition was obtained, even if the lichenin solutions were allowed to stand for as long as a week (in a refrigerator). This is clearly shown in Fig. 10. It will be seen, too, that the initial rate of decomposition was again reached on a

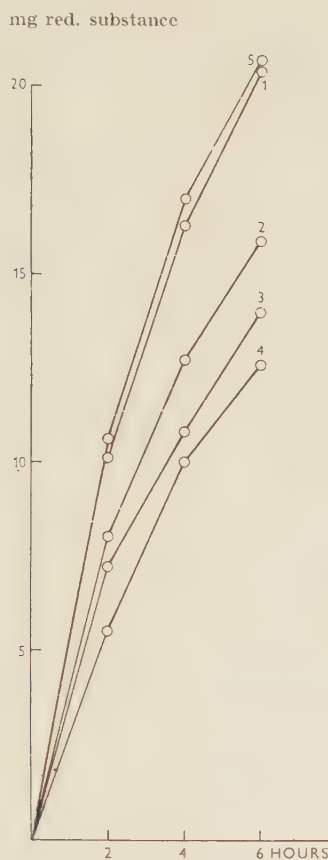


Fig. 10. Hydrolysis of a lichenin solution of increasing age. The solution was 1. freshly prepared, 2. 1 day old, 3. 2 days old, 4. 7 days old, and 5. 7 days old, boiled a second time. pH 7.0. 10 mg H-preparation¹ in each flask. 2 parallel flasks.

renewed boiling, which corresponds to the findings of GRASSMANN and associates.

Considering my own results it seemed to be more convenient to use a fresh lichenin solution, even though the shape of the curves in the later stage may perhaps be influenced by the "aging" of the substrate. The reduction in the rate of hydrolysis is about 20 per cent in a solution which is 24 hours old. GRASSMANN and associates noted a decrease amounting to 30 per cent during the same period.

The cause of the "aging" probably lies in a reduction of the dispersity of the lichenin on the standing of the solution. The greater particles formed

¹ Cf. footnote 1 on p. 81.

are naturally not so easily attacked by enzymes. This fact leads us to the problem of the constitution of lichenin and the relationship between lichenin and true cellulose.

As yet there exists much uncertainty on this point. KARRER, JOOS and STAUB (1923) compared cotton cellulose with lichenin and established identity in many respects. The main difference seemed to lie in the dispersity, which enzymically is of the greatest importance. HERZOG (1926) observed that the X-ray diagram of lichenin diverged from that of native cellulose and agreed more with that of regenerated cellulose. According to CARTER and RECORD (1939), lichenin consists of unbranched chains with a degree of polymerization of 80—160¹. HESS and LAURIDSEN (1940) suggested that the linkages between the glucose units of the lichenin might be in 1,1- and 4,4-position, but this is doubtful from enzymatic points of view. DRAKE (1943) has also rejected this hypothesis in view of data obtained at acid hydrolysis of lichenin. PERCIVAL and GRANICHSTÄDTEN (1942) have obtained results which indicate that the primary alcohol groups of cellulose are shielded by some sort of cross linkages, while those of lichenin are not.

KARRER and STAUB (1924 p. 928) found a small amount of phosphorus in lichenin, which may be of significance.

Regarding the cellophane samples used in the experiments, see below p. 111.

The cellobiose used was the commercial preparation "Pfanstiehl" c. p.

Other substrates occasionally tried are described in connection with corresponding experiments.

3. Arrangement of the enzyme experiments. Sugar determinations.

The enzyme experiments were made in 50 ml Erlenmeyer flasks of Jena or Pyrex glass. Ordinarily the following substrate was used: 5 ml M/15 phosphate buffer of pH 7.0 + 5 ml 0.7 per cent lichenin solution. The flasks then contained 32 mg dry lichenin (corresponding to 36 mg glucose) in a M/30 phosphate buffer. As a disinfectant 2 ml toluene were added. In the cellophane experiments 100—500 mg cellophane and 5 ml of water were added instead of lichenin.

After addition of the dry bacteria or the enzyme solution to be tested, the flasks were plugged with rubber stoppers and shaken vigorously. They were placed in a rack in a thermostat bath at 36° C and were submitted to continuous shaking. The equipment used has been figured by NILSSON (1941).

For analysis 2 ml samples were taken out at intervals, as a rule after 2 and 6 hours, sometimes also after 4 or 24 hours. In the lichenin experiments, no change in concentration occurred through this sampling. On the

¹ Unpublished results by MOSIMANN indicate that branched chains with a D. P. of up to 500 may exist (determined in the ultracentrifuge).

other hand, when cellophane was used as a substrate the concentration increased gradually through the sampling. In most later experiments with cellophane this was, therefore, cut into a number of equal strips, an aliquot part being withdrawn at each sampling. These strips could also be used for examination of the reduction in tensile strength of the cellophane.

As a rule the method of WILLSTÄTTER and SCHUDEL (see BAMANN and MYRBÄCK 1941 p. 1077) was used for determination of reducing substance. It is true that this method is less specific in respect to sugar than the copper methods, but it is stoichiometric and has the advantage of being simple to handle when many samples have to be analyzed. The indications given by MYRBÄCK and ÖRTENBLAD (1938) were considered, when using this method. As only glucose was concerned, the extreme precautions necessary when determining maltose, for instance, were not required. In detail my determinations were carried out as follows.

3 ml amounts of 0.1 N iodine were pipetted into 100 ml Erlenmeyer flasks (preferably with ground glass stoppers) before each sampling. At the sampling 2 ml were pipetted from each flask into the corresponding flask with iodine. 4.5 ml 0.1 N NaOH were then slowly added from a burette with a glass stopcock, while the flasks were shaken continuously. The addition took at least 1.5—2 minutes for each flask. About 20 minutes after the addition of the NaOH the iodine was liberated through addition of 5 ml 0.1 N sulphuric acid. For the titration 0.02 N thiosulphate was used with 0.5 ml 1 per cent starch solution as indicator. A very distinct end-point was obtained.

In most experiments two parallel flasks were set up. Since the values obtained generally agreed very well and the space available in the water bath was limited, sometimes only one flask was set up, the time curve obtained giving a satisfactory picture of the course of hydrolysis. All values plotted in the figures are calculated as glucose and given as mg reducing substance. The reducing value of the enzyme preparation and the lichenin was subtracted before calculation of the glucose quantity. 10 mg of the preparation as a rule contained 2—3 mg reducing substance, calculated as glucose.

C. The Hydrolysis of Lichenin.

1. Environmental factors influencing the rate of hydrolysis.

Influence of temperature.

Considering the optimal temperature for living bacteria (32—37° C), the temperatures 30, 36 and 42° C were tried. The results are seen from the accompanying figure 11.

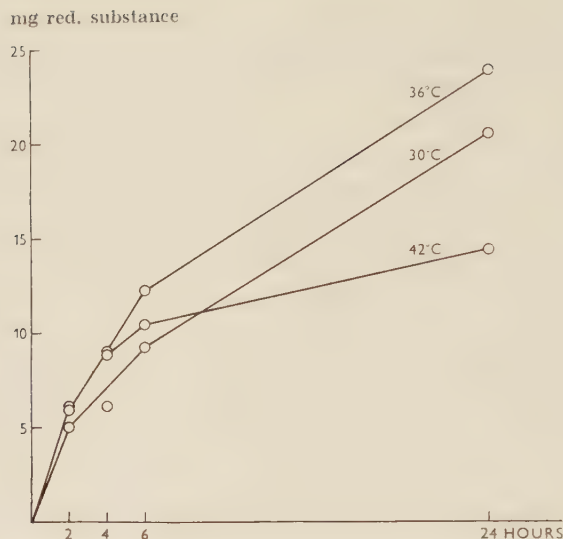


Figure 11. Hydrolysis of lichenin at 30, 36 and 42° C. pH 7.4. In each flask 10 mg H-preparation¹. 2 parallel flasks.

As is readily seen the lichenin was broken down most rapidly at 36° C. At 42° C the speed was equally great in the first stage but was later considerably reduced. This is very probably due to a destroying effect exerted by the high temperature on the enzyme system (a destruction of the cellobiase alone would give such a curve also). From a later experiment it was, in fact, evident that the enzyme system concerned is very sensitive to higher temperatures. 10 mg dry bacteria preparations were heated for 5 minutes in a phosphate buffer of pH 7.0 to temperatures from 50 to 80° C and were then used directly for an enzyme experiment. The result of this experiment will be seen from Fig. 12. It was evident that even a temperature of 50° C, acting on the bacteria preparations for 5 minutes only, reduced the activity of the preparations to about half. A prolonged treatment at 42° C, as in the preceding experiment, will thus probably have an injurious effect on the enzymes concerned. KARRER and associates (1924 p. 144) found that the *Helix* lichenase is sensitive to temperatures exceeding 40° C (3 hours' treatment). In remarkable contrast to this stands the wide range reported by PRINGSHEIM (1912 p. 273) for the cellulase in thermophilic bacteria. This cellulase appears to be active within a temperature range of 50 degrees (20—70° C).

As a consequence of the above experiment all following enzyme studies with lichenin have been carried out at 36° C.

¹ See footnote 1 on p. 81.

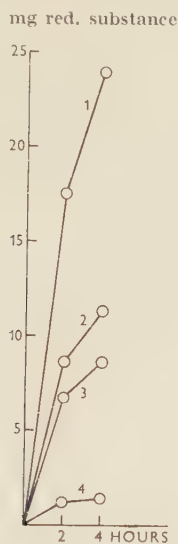


Fig. 12. Influence of heating on the activity of *Cytophaga* preparations. 1. Not heated, 2. heated to 50°, 3. to 60° and 4. to 70° C. 10 mg C-preparation.

Influence of pH.

The action of the *Helix* lichenase has an optimum at pH c. 5 (KARRER, JOOS and STAUB 1923). GRASSMANN and collaborators (1933 a) found pH 4.5 (4.0—5.0) optimal for the action of the *Aspergillus* lichenase (in acetate buffer). When studying the enzymes separated from the luizym mixture, FREUDENBERG and PLOETZ (1939) found the optima of 5.9 for the lichenase activity and of 4.7 for the cellulase activity.

The growth optimum of *Cytophaga* suggested that a pH of about 7 would be optimal and, that being so, it seemed natural to try a phosphate buffer. In order to determine the optimum accurately a pH experiment was set up. The variation in pH was effected by addition of varying amounts of primary and secondary phosphates according to SÖRENSEN. In this way a series from about 5 to 8 was obtained. During the course of the hydrolysis the pH values changed to a certain extent:

	Initial pH	Final pH (24 hours)
1.	5.2	5.2
2.	6.1	6.2
3.	7.0	6.6
4.	7.4	7.3
5.	7.8	7.3
6.	8.1	7.5

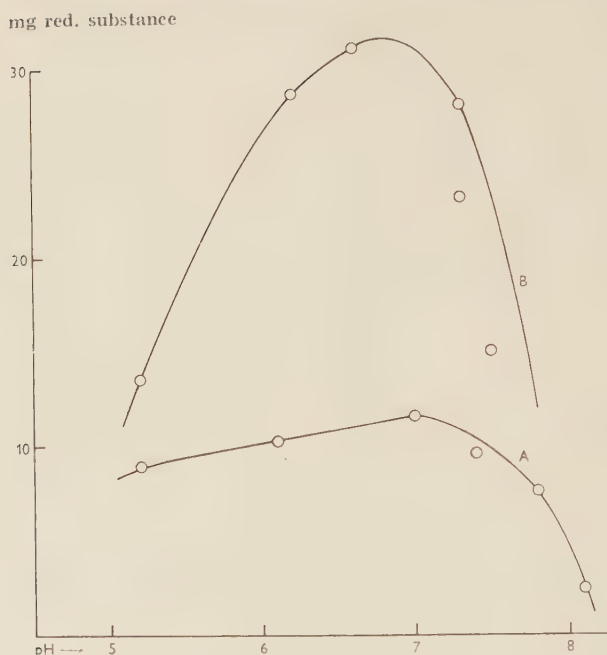


Figure 13. Hydrolysis of lichenin at various pH values. A: after 2 hours. B: after 24 hours. 20 mg H-preparation. 2 parallel flasks.

The result of the experiment will be seen from the accompanying figure (fig. 13).

From the curves it will be seen that the optimum range for glucose formation from lichenin lies between pH 7.5 and 5.5 with a weak peak at pH 7.3—6.2. 7.8 (7.3 after 24 hours) is apparently too high and 5.2 too low. Thus the *Cytophaga* lichenase seems to be in this respect somewhat different from the lichenases from other sources mentioned above. The results must, of course, be evaluated with some caution, since the presence of impurities in the preparations may have influenced the results. This is true also of other experiments recorded in the following pages.

Although the lichenase in *Cytophaga* is not active outside rather narrow limits, as seen from the preceding experiments, the tolerance of the enzyme in question to high and low pH values is fairly great. Results reported later (pp. 102 and 105) show that the activity is not destroyed by treatment at pH 3 or 9.

As a consequence of the results obtained in the pH experiments all subsequent experiments were carried out with a phosphate buffer of pH 7.0.

Influence of phosphate.

An experiment was set up in order to compare the rate of hydrolysis in samples without phosphate and with increasing amounts of phosphate buffer of pH 7.0. In some flasks with 1/300 M phosphate an addition of chalk was made in order to counteract pH changes. The result of the experiment is seen in Fig. 14.

As it might be suspected that a certain change in pH would take place especially when low concentrations of phosphates were present, pH determinations were made at the times 0 and 24 hours. No differences were noted, however.

From the curves some evidence is obtained that phosphate has a stimulating influence on the lichenin hydrolysis. No greater differences exist, but probably even the samples without phosphate being added contain phosphate derived from the bacteria preparation. In all subsequent experiments 1/30 M phosphate was used. As mentioned above (p. 80), GRASSMANN and associates found that phosphate stimulated the hydrolysis of lichenin. SIMOLA (1931 b) found a M/30 phosphate buffer optimal for the cellulose hydrolysis by *Cellulobacillus myxogenes*.

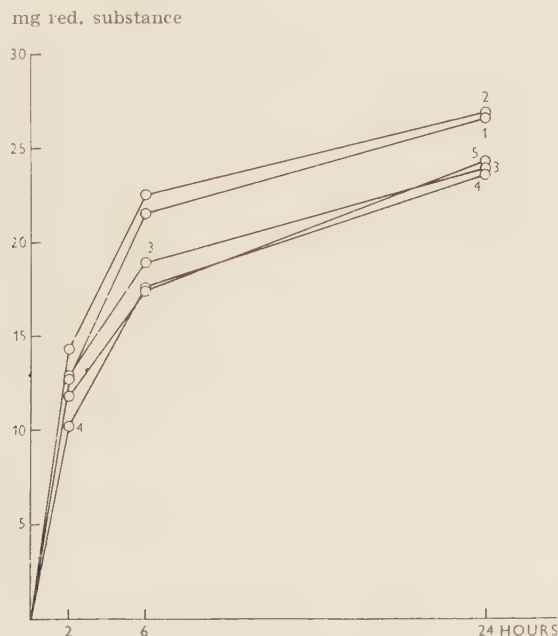


Figure 14. Hydrolysis of lichenin at various phosphate concentrations. Flasks containing 1. 1/30 M phosphate, 2. 1/100, 3. 1/300 4. 1/300 M + chalk, 5. Without phosphate. 20 mg H-preparation. 2 parallel flasks.

Determinations of phosphate were carried out by me in order to ascertain whether some phosphate was perhaps fixed in organic bond. The determinations were made according to the method of EMBDEN (see BAMANN—MYRBÄCK 1941 p. 1610) on 2 ml samples withdrawn after 24 hours. The samples were added to 10 ml amounts of 10 % trichloroacetic acid, and the filtrates were used for phosphate determinations. The results will be seen from Table 13.

Table 13. Amount of Inorganic Phosphorus Present after Hydrolysis of Lichenin by a *Cytophaga* Preparation.

Sample with	Amount of P_2O_5 found mg	Amount of P_2O_5 after subtraction of blank mg	Amount of P_2O_5 calculated mg
M/30 phosphate.....	25.2	23.9	23.7
M/100 »	8.3	7.0	7.1
M/300 »	3.8	2.5	2.4
M/300 » + chalk	3.8	2.5	2.4
Without phosphate (= blank).....	1.3	0	0

From this the conclusion may be drawn that all the phosphate added is present as inorganic phosphate. No evidence is thus obtained that the phosphate has partaken directly in the decomposition of the lichenin.

STANIER (1942 b p. 177) suggested that the decomposition of cellulose might be a phosphorolytic one, as mentioned earlier (p. 73). In order to examine this possibility further, I started a second experiment, in which in addition to phosphate Mg-ions, NaF and adenylic acid also were present. Each flask received the following amounts: NaF 8.4 mg (= 20 mM), $MgCl_2 \cdot 6 H_2O$ 16.2 mg (= 8 mM), muscle adenylic acid 4 mg.

Mg-ions and adenylic acid function as coenzymes for certain phosphorylase systems, NaF inhibits the action of the phosphatases.

Determinations of inorganic phosphorus were carried out according to EMBDEN, as in the preceding experiment, but now after 1 hour and 6 hours. The phosphate was, however, totally recovered as inorganic phosphate in all samples. Thus the result agrees with that in the preceding experiment. It must, however, be admitted that a hypothetical phosphorylase may have been destroyed at the preparation of the bacteria.

The hydrolysis of lichenin in this experiment had, however, a somewhat different course in the different samples. This will be seen from the accompanying curves (Fig. 15).

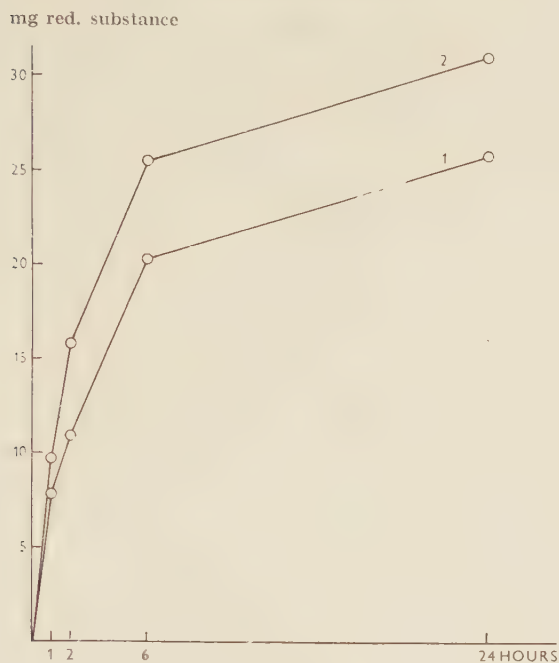


Figure 15. Hydrolysis of lichenin in 1/30 M phosphate buffer. 1. Without and 2. with the addition of MgCl_2 , NaF and adenylic acid. 20 mg H-preparation. 2 parallel flasks.

As is evident, the addition of the substances mentioned had a remarkable effect on the rate of the lichenin hydrolysis. Since the mixture only was tested, it could not be decided from this single experiment whether the effect was a combined one or whether some of the substances added were responsible for the effect obtained. This has been further studied in later experiments.

Influence of various ions.

The preceding experiment invited studies into the influence of various inorganic salts on the lichenin hydrolysis. As a control of the preceding experiment first the effect of MgCl_2 and NaF, taken separately and together, was examined. The quantities used were the same as in the preceding experiment. The result is seen from Fig. 16.

From this experiment it seems obvious that the effect observed is due to the presence of MgCl_2 . From later experiments, where MgSO_4 also was tested, it is evident that Mg and not the chloride ion was the stimulating agent in the hydrolysis process.

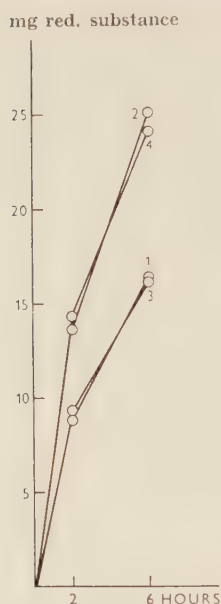


Figure 16. Hydrolysis of lichenin in the presence of MgCl_2 and NaF . 1. Without addition, 2. + MgCl_2 , 3. + NaF , 4. + MgCl_2 and NaF . 20 mg H-preparation. 2 parallel flasks.

In certain experiments with other *Cytophaga* preparations no such effect of Mg was found. This may be due to the Mg content in the preparations used. Since the nutrient medium from which the preparations have been

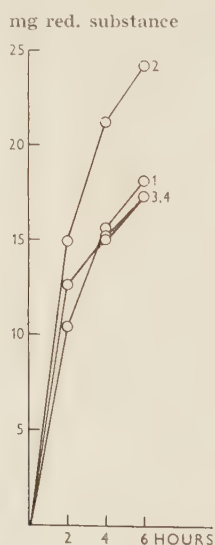


Figure 17. Hydrolysis of lichenin in the presence of Mg, Ca and Mn. 1. Without addition, 2. + MgCl_2 , 3. + CaCl_2 , 4. + MnCl_2 . 20 mg H-preparation. 2 parallel flasks.

produced, contained Mg, it might well be that a small amount of Mg was present in the preparations in certain cases.

In an experiment some ions other than Mg were also tested, namely Ca and Mn. The concentration was 5 mM of the respective chlorides. This corresponds to 1.2 mg Mg, 2.0 mg Ca and 2.7 mg Mn in each flask. Fig. 17 shows the resulting values.

The results indicate that only Mg, and not Ca or Mn, had a markedly positive effect. Regarding these elements it is also true that the preparations used may have contained traces of them. Thus it is not impossible that by using a purified enzyme preparation other results may be obtained.

Influence of the antisepticum.

As it might be suspected that the addition of toluene to some extent influenced the results of the enzyme experiments, this was omitted in some samples in an experiment. As a control 2 ml toluene were added in other samples in the usual way. As a comparison chloroform was also tried as antisepticum. It is clear that in experiments of long duration some sort of antisepticum has to be added to prevent infection, but during the short period of 6 hours used in the experiment reported here, a notable activity of foreign bacteria or moulds probably cannot take place. The result of the experiment will be seen from Fig. 18.



Fig. 18. Hydrolysis of lichenin in the absence of antisepticum (1) and in the presence of toluene (2) or chloroform (3). 20 mg H-preparation. 2 parallel flasks.

As will be seen from the Figure, both antiseptics had a somewhat retarding influence on the hydrolysis. Chloroform was more inhibiting than toluene. The results speak against the possibility of an infection, since a development of contaminating bacteria would very probably have resulted in a consumption of glucose.

2. Discussion of some further factors influencing the rate of hydrolysis.

By using a large amount of bacteria preparation, the rate of lichenin-splitting naturally becomes increased. There does not seem to exist a direct proportionality between enzyme quantity and rate of hydrolysis, however. As mentioned above, KARRER, JOOS and STAUB (1923) found a fairly constant proportion (1.5—1.6, with a purified lichenase 1.4) between the amount of lichenin split by the simple and double enzyme quantity respectively. A similar result was obtained by the present writer in an experiment (see Table 14).

Table 14. Influence of Increasing Amounts of Bacteria Preparation on the Rate of Lichenin Hydrolysis.

Hours	Mg glucose formed by mg preparation				Proportions
	40	20	10	5	
2	14.6	9.2	5.5	3.3	1.6, 1.7, 1.7
6	22.8	17.9	10.3	6.1	1.3, 1.7, 1.7
24	28.4	28.7	17.8	11.2	1.0, 1.6, 1.6

The course of the lichenin-splitting can be more clearly seen in the accompanying figure (Fig. 19).

From the Table it is evident that the value of 1.6—1.7 is fairly constant up to a decomposition of c. 50 per cent (18 mg glucose formed). Too much significance should not be attached to these values, however. The enzyme preparations were not purified, and it has been shown (KARRER, JOOS and STAUB 1923 p. 812, FREUDENBERG and PLOETZ 1939) that the degree of purity of the enzyme preparation may be of influence. The purity of the lichenin may also affect the results (PRINGSHEIM and BAUR 1928). GRASSMANN and his associates (1933 a) obtained a direct proportionality between enzyme quantity and amount of substrate converted, as mentioned above on p. 80.

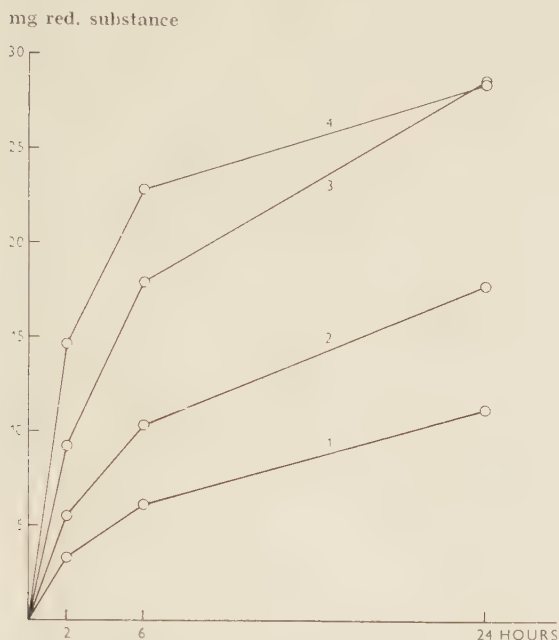


Fig. 19. Hydrolysis of lichenin by increasing amounts of bacteria preparations.

Amounts added: 1) 5 mg, 2) 10 mg, 3) 20 mg, 4) 40 mg H-preparation.

2 parallel flasks.

As is seen from the figures in the preceding pages the shape of the curves is substantially the same. The hydrolysis proceeds fairly rapidly in the first stage and is then gradually retarded. Possibly it follows first the course of a monomolecular reaction, as shown for *Helix* lichenase by KARRER and collaborators (1923), but the determinations made by me do not permit any such conclusions to be drawn. On the basis of the values given

in Table 14 no constant value can be obtained for $\frac{1}{t} \cdot \log \frac{a}{a-x}$. The fall-

ing off of the curves may be due to the decreasing concentration of lichenin in the solution, but other factors may also influence the rate of hydrolysis.

Among these the aging of the lichenin, previously mentioned, must be recalled.

A further important detail is the glucose gradually formed in the flasks. Finally it must be pointed out that part of the glucose may be converted to further degradation products, ultimately carbon dioxide. The last two possibilities have been subjected to examination.

Since each flask contained about 32 mg lichenin, theoretically about 36 mg glucose could be formed during the hydrolysis. Such a complete

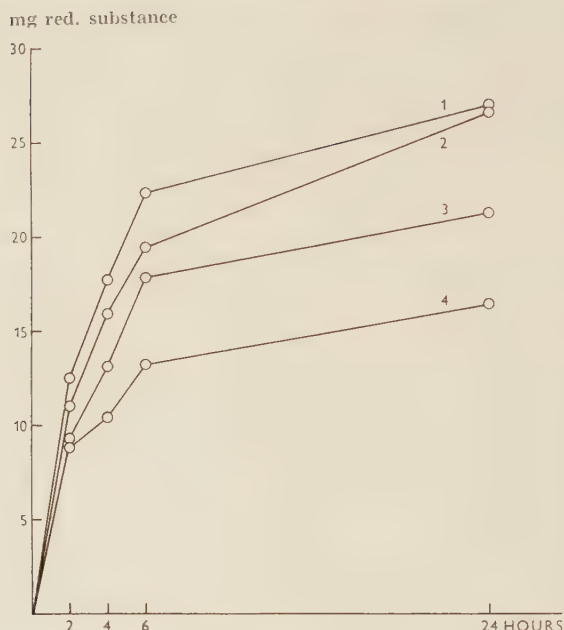


Figure 20. Hydrolysis of lichenin on addition of increasing amounts of glucose. 1) No addition, 2) 0.1 % glucose, 3) 0.25 %, 4) 0.5 %. 10 mg H-preparation.

hydrolysis has never been attained, but in several cases about 30 mg glucose were formed. This represents 0.3 per cent. It had, however, already been shown by KARRER and collaborators (1924 pp. 144, 916) that glucose and other sugars to some extent inhibit the action of the *Helix* lichenase even in a 0.1 % concentration. The glucose caused a reduction of the rate of hydrolysis to about 60 % of the value in the absence of sugars (4 hours' incubation).

In usual growth experiments with *Cytophaga* 0.3 % glucose is a strongly inhibiting concentration, as pointed out earlier (p. 43).

An enzyme experiment was set up in order to examine the influence on the hydrolysis of increasing amounts of glucose added to the flasks. The result of this experiment is seen from Fig. 20.

As seen from the curves, glucose in fact exhibits a retarding influence on the hydrolysis process. Even the highest concentration used (0.5 %) permits, however, a considerable hydrolysis to take place. The complete inhibition of the cellulose decomposition by *Cytophaga* on addition of glucose, as discussed earlier (p. 49), thus cannot be explained as an inhibition of the hydrolyzing enzyme system. Compare the experiment with cellophane on p. 115.

It might be suspected that at least part of the glucose was further converted to other degradation products. In this case naturally all the lichenin could not be recovered as glucose. In order to examine this possibility an experiment was set up, the CO_2 evolved being measured in fermentation burettes (see NILSSON 1941). The glucose formed was determined in the usual way after terminating the experiment after a period of 4 hours. 31 mg glucose were then found in the solution. The evolution of CO_2 was measured continuously during the whole course of the experiment and amounted to 1.5 ml after 4 hours (20 mg C-preparation, without toluene).

This is a quantity which roughly corresponds to the amount of lichenin not accounted for by the glucose recovered. This is true on the assumption that the glucose is converted according to the formula for anoxydative fermentation: $\text{C}_6\text{H}_{12}\text{O}_6 = 2 \text{CO}_2 + 2 \text{C}_2\text{H}_5\text{OH}$.

It must, however, be pointed out that the experiment mentioned is very preliminary and that it is only an attempt to explain the glucose deficit. Some experiments in which the evolution of CO_2 was studied further, have given very inconsistent results. It is possible that the kind of preparation may influence the result. It seems, however, probable that in many cases, when the lichenin is converted to glucose to a less degree than would be expected from the amount of preparation present, the explanation is as has been assumed above, namely that the remainder, not accounted for, is further converted to CO_2 . Whether the glucose is converted in an oxydative or anoxydative manner cannot yet be decided.

The enzyme system converting glucose to simpler compounds is obviously to a great extent, but not totally, checked at the conditions prevailing in the experiments. It is, however, also possible that the *Cytophaga* enzymes responsible for the decomposition of glucose are almost entirely destroyed in the preparation of the bacteria. Certain results with preparations which were not dried, might be interpreted in such a way.

3. Improvement of the efficiency of the dry preparations.

Through variation in the methods of culturing *Cytophaga* and preparing bacterial substance attempts were made to increase the yield and activity of the preparations. In the following pages some experiments in this direction are reported.

Influence of aeration.

In an experiment four series of flasks, each containing six parallels, were inoculated with *Cytophaga globulosa*. The vessels used were:

1. One litre Erlenmeyer flasks with 300 ml medium, not aerated.
2. As in 1., but flasks aerated.
3. One litre Erlenmeyer flasks with 700 ml medium, aerated.
4. 1.4 litre Kolle flasks with 500 ml medium, not aerated.

As substrate cotton-wool was used. The cultivation took place in an incubator at 30° C.

In the aerated flasks and the Kolle flasks growth was evident after 4 days. The Erlenmeyer flasks without aeration did not show any obvious growth until 6—7 days after inoculation.

The experiment was terminated after 16 days. From the filtered culture liquid the bacterial cells were precipitated by addition of HCl. The precipitate formed was centrifuged, and the sediments were collected and neutralized with 1 N NaOH. They were distributed over the surface of large, glass Petri dishes and dried in a refrigerator. The bacteria were scraped off after drying as completely as possible and weighed for each flask separately. The amount of decomposed cellulose was also determined for each flask and in this way the proportion between cellulose decomposition and formation of bacterial substance could be calculated.

The values obtained in the analyses will be seen from Table 15.

The preparations were combined for each series and used for an enzyme experiment. The result is seen from Fig. 21.

As will be seen from the Table and Figure, the aeration not only resulted in a far greater yield of bacterial substance, but the beneficial effect is even more emphasized by the higher activity of the preparations

Table 15. Decomposition of Cellulose and Yield of Bacteria in Flasks with and without Aeration.

The Values of the Table are Mean Values of 6 Parallels.¹

Method of culturing	1.	2.	3.	4.
Original cellulose mg	1 432	1 432	3 341	2 387
Cellulose decomposed mg	337 ± 9.1	1 024 ± 24	1 817 ± 90	728 ± 26
Percentage	24	72	54	31
Yield of bacteria mg	90 ± 5.8	328 ± 6.9	421 ± 16	201 ± 9.8
In % of decomposed cellulose . . .	27	32	23	28

¹ The mean errors given in Tables 15 and 17 are calculated according to the formula

$$m = \sqrt{\frac{S(x - \bar{x})^2}{n(n-1)}}$$

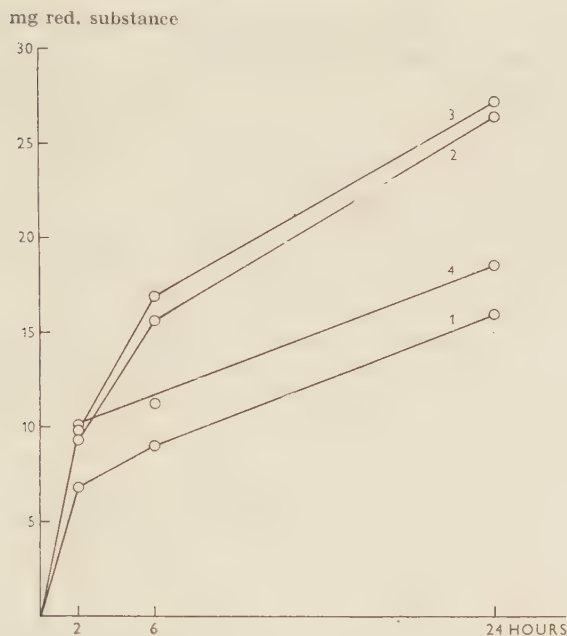


Figure 21. Hydrolysis of lichenin by means of bacteria preparations obtained after culturing in different ways. 1, 2, 3, 4: see p. 98. 20 mg H-preparation. 2 parallel flasks.

obtained. The great increase in yield resulting from aeration is a phenomenon generally met with in bacteria (cf. WINSLOW *et al.* 1932, RAHN and RICHARDSON 1942).

Culturing in a larger volume (700 ml) with aeration gave the highest absolute yield of bacterial substance. Calculated in respect to the cellulose consumed, however, the yield is not so great as in flasks with a smaller liquid volume. Because of extensive frothing in the case of aeration the use of large volumes is not convenient and smaller ones have been used in further experiments.

The Kolle flasks offer no advantages and are difficult to handle. They have consequently not been used later.

Since the importance of good aeration became established, all subsequent cultures for production of bacteria preparations have been arranged with aeration.

In the above experiment the air was let in through tubes to the bottom of each flask. It is clear that this is not an entirely satisfactory arrangement. Perhaps in this case the main effect of the aeration is the removal of carbon dioxide from the liquid and the gas phase above the surface, but no effective stirring of the fluid takes place. A better aeration, simultane-

ously involving a mechanical stirring of the medium, is obtained by the use of fine grade porous plates, as in Kluver flasks or other vessels equipped with special air distributors. In this case the time of incubation may also be reduced considerably.

A number of experiments have been carried out using such arrangements. In order to get larger amounts vessels of stainless steel with a volume of c. 25 litres, containing 12 litres of medium, have been used. Through a hole in the bottom air has been let in, the air being distributed by passing a filtering cylinder of porous ceramics. The cellulose, in most cases cotton-wool, was dispersed in the liquid over a perforated plate under which the filtering cylinder was placed. In such a way the cotton-wool did not sink below the level of the air inlet and was entirely penetrated with fine air bubbles. The greatest difficulty, when using such vessels, was the sterilization, which must be most carefully done. For elimination of the frothing, a foam-inhibiting fat has sometimes been added.

In the Kluver flasks the rate of cellulose decomposition was very high. In some experiments the following values were obtained after 5 days' incubation at 30° C.

Table 16. Decomposition of Cellulose in Kluver Flasks.

Substrate	Original cellulose g	Residual cellulose g	Decom- position, per cent
1. Cotton-wool	4.78	2.58	46
2. Cellulose-wadding	4.72	2.52	47
3. Cotton-wool	4.78	1.47	69
4. Cotton-wool	4.78	2.71	43
5. Cotton-wool	4.79	1.31	73
6. Cotton-wool	4.79	1.03	78

The bacterial substance was in these cases prepared in different ways and the activities determined are therefore not directly comparable. The activity was good as a rule.

Influence of the source of cellulose.

In an experiment cotton-wool and cellulose-wadding were compared as substrates in the production of bacteria preparations. Concurrently 300 and 500 ml volumes of medium with 0.5 % of cellulose were set up. The flasks were all aerated at a temperature of 33° C. The experiment ran for 17 days. After that the culture liquids were filtered, the filtrates combined and precipitated with HCl. Two parallel samples (100 ml) from

each series were then withdrawn and centrifuged, neutralized and dried separately. Cellulose determinations were carried out for each flask separately.

In the following Table (17) the values for cellulose decomposition and formation of bacterial substance are given.

Table 17. Decomposition of Cellulose and Yield of Bacteria at Cultivation on Different Cellulose Sources.

5 Parallel Flasks.

Substrate	Cellulose-wadding		Cotton-wool	
Original cellulose, mg	2 360	1 415	2 390	1 430
Cellulose decomposed, mg.	845 ± 53	592 ± 48	1 098 ± 98	771 ± 42
Yield of bacteria in 100 ml, mg	24.6, 24.8	27.1, 29.3	38.8, 41.8	39.9, 40.8
Yield of bacteria per flask, mg .	106	73	173	104
In % of cellulose decomposed. . .	12.5	12.3	15.8	13.5

The amount of bacterial substance given in the Table above was obtained after drying at 40° C. For a study of the enzyme activity new samples were taken from the remaining undried precipitate. These samples were dried *in vacuo* and used for an enzyme experiment. 10 mg of preparation were used. In this no significant differences were obtained, however, and no curves from this experiment are therefore given. 10 mg preparation gave after 2 hours c. 16 mg, after 4 hours c. 21 mg and after 6 hours c. 24 mg of reducing substance.

Although the variation of the values for cellulose decomposition is very great, it seems possible to draw the conclusion that cotton-wool is more favourable as substrate, since a higher yield of bacterial substance is obtained than with cellulose-wadding as substrate. This seems mainly to be due to a more vigorous decomposition of cotton-wool than of cellulose-wadding. It is worth mentioning that NORMAN and BARTHOLOMEW (1940) have obtained greater amounts of "gum" on cotton than on pulp (l. c. Tab. 3).

Other possible sources of cellulose, as for instance ground filter paper, have been tried as substrates, but have not given better results.

Variation of the methods for the preparation of bacterial substance.

"H-preparations." Ever since it was discovered that bacteria preparations of *Cytophaga globulosa* could be obtained through precipitation

with acid¹, this method has been examined in some detail in order to improve the yield and activity of the preparations. It is in fact a most simple method, requiring no distillations *in vacuo* or a prolonged dialysis. A considerable drawback, however, is that this method could be used for *Cytophaga globulosa* only. For that reason *C. globulosa* has been used for most enzyme experiments.

In the beginning no special precautions were taken at the precipitation with acid, and the activity of the preparations obtained was relatively weak. The effect of the dry preparations obtained can be seen for instance from Figs. 15—19. In these experiments 10 mg dry preparation yielded about 6 mg reducing substance after 2 hours and 20 mg dry preparation about 10 mg after 2 hours.

The activity remains undiminished for a long period. In Fig. 17 above the preparation used was about 2¹/₂ months old.

It was found that the activity of the preparations obtained through precipitation with acid could be considerably increased, if the preparatory work was performed rapidly and at a low temperature.

In this case the whole culture liquid had to be centrifuged, whilst earlier the precipitate was allowed to settle overnight or at least for some hours, after which most of the supernatant liquid could be poured off without any precipitate being lost. Before precipitation the culture liquid was cooled down to 0° C. HCl was added dropwise until flocculation took place (pH 3—3.5), whereupon the liquid was centrifuged. The centrifugation was carried out in an angle-head centrifuge of 3 l. capacity as rapidly as possible. After pouring off the supernatant liquid the sediments were combined and neutralized immediately with NaOH.

A great improvement of the dry preparations was obtained by means of the freezing and thawing technique. The result from an experiment with a preparation frozen and thawed five times is given in Fig. 22.

It seems evident that the freezing out has improved the effect of the preparation considerably. The unfrozen preparation is rather weak, giving only 8 mg reducing substance after 2 hours, the frozen preparation giving 15 mg. A dry preparation from another experiment, but treated in a similar way, gave in 2 hours 22 mg reducing substance (20 mg preparation).

Later experiments gave even more active preparations, 5 mg dry bacteria giving more than 15 mg reducing substance in 2 hours. Such preparations had been cautiously produced with the procedure described above. Results with frozen preparations are also to be found in Figs. 27 and 29 (cellophane experiments).

¹ Preparations obtained by means of precipitating with acid have been called H-preparations.

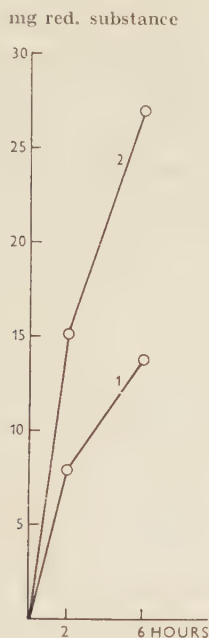


Fig 22. Hydrolysis of lichenin by a frozen and thawed preparation. 1) Untreated dry preparation, 2) Preparation frozen and thawed 5 times before drying. 20 mg H-preparation.

During a repeated freezing and thawing a very viscous liquid is excreted by the bacterial cells. Simultaneously the bacterial substance shrinks. The cells and the liquid can be separated by centrifuging, although no perfectly clear solution is obtained. This solution is very active against lichenin, and also cellophane. It has been used in certain cases, for instance in the experiment with cellophane reported on p. 72.

In some experiments the effect of precipitating with other acids than HCl was investigated. Thus H_2SO_4 , H_3PO_4 and some organic acids were studied, namely formic, acetic, lactic, oxalic and citric acid.

Equal volumes (100 ml) of a *Cytophaga* filtrate were precipitated with HCl, H_2SO_4 and H_3PO_4 . The preparing was carried out in the usual way. After drying, the preparations were weighed. They were also used for an enzyme experiment. In Table 18 the figures obtained are given.

It would appear from the Table that H_3PO_4 and H_2SO_4 have given somewhat higher yields of bacterial substance than HCl, which is perhaps due to a certain precipitating action of the bivalent and trivalent ions in these acids. On the other hand, a higher activity was registered in preparations obtained after precipitation with HCl. This may possibly be ex-

Table 18. Amounts of Bacteria Preparations obtained after Precipitation with Various Acids and the Effect of such Preparations.

The Values from the Enzyme Experiment are Mean Values from 2 Parallel Flasks.

Precipitant	Mg bact. substance from 100 ml medium	Mg red. substance formed by 15 mg preparation	
		2 hours	6 hours
H ₃ PO ₄ pH 3	52.2	10.2	18.1
H ₂ SO ₄ pH 3	52.8	7.7	14.7
H ₂ SO ₄ pH 4	41.0	10.5	17.6
HCl pH 3	47.5	13.4	25.0
HCl pH 3.5	44.0	12.6	22.4
HCl pH 4	41.0	11.4	19.4

plained by assuming that Cl ions stimulate the hydrolysis. This possibility has, however, not yet been examined.

The precipitation seems to be less complete at pH 4 than at pH 3.

As mentioned above, some organic acids were also tried in this respect. But because of the weakness of the acids named, especially of acetic acid, as a rule no complete precipitation occurred until great amounts of acids were added. Only the substance precipitated with formic acid has been tested in an enzyme experiment. From 100 ml 51.5 mg substance were obtained, but 10 mg of this precipitation gave only 4.4 mg reducing sugar in 2 hours. The effect was thus very weak. An HCl-preparation tested concurrently gave 12.9 mg reducing sugar in 2 hours.

Apart from the method reported above, others (precipitation with methanol, ethanol, tannin, ammonium sulphate, etc.) have been tried in order to obtain active dry bacteria.

Since none of these methods offered obvious advantages over the precipitation with acid in the work with *Cytophaga globulosa*, the latter method has been preferred. Sometimes, especially when working with other *Cytophaga* strains, a concentration of the culture liquid *in vacuo* was made in order to obtain active bacteria preparations.¹

This concentration was carried out in the "Vacuum-Umlauf-Verdampfer" of the firm of Schott und Genossen in Jena. About 1.6 litres could be evaporated per hour at a temperature of 20—25° C. After concentrating to about 1/30 of the original volume (10 litres concentrated to 300 ml) the solution was dialyzed in a cellophane tubing to remove the salts. It was observed that the cellophane was attacked by the bacteria even at 0° C on prolonged dialysis. Therefore some precautions had to be taken and the tubing, if necessary, changed. The undissolved salts present in the concentrated culture fluid could generally be removed through a brief centrifugation (5 min. at

¹ Compare footnote 3, p. 81.

3 000 rpm). A final concentration in a usual vacuum distillation apparatus gave a product mainly consisting of bacterial cells, although a certain amount of salts was inevitably present. The yield and activity of such preparations were good as a rule. Irregularities in this respect occurred and might perhaps be explained by the varying content of salts in the preparations. The activity was as a rule higher than that of the H-preparations, but later when the effect of the latter preparations was improved considerably, the preparing of bacteria through concentration *in vacuo* offered no advantage when working with *Cytophaga globulosa*.

Experiments with C-preparations are shown in Figs. 12 and 23.

4. Extraction of the crude preparations and experiments with cell-free solutions of the cellulose-decomposing enzyme system.

Through extraction of the dry bacteria, it was possible to demonstrate that the cellulose-decomposing enzyme system is readily brought into cell-free solution.

In the extraction experiments as a rule 100 mg of a dry preparation were extracted for varying periods with 20 ml water or buffer solution with or without addition of toluene. Thus 1 ml roughly corresponded to 5 mg dry preparation. After the period desired the samples were centrifuged for 15 minutes in a Corda centrifuge and the turbid solution was filtered through a bacterial filter ("G 5 on G 3" from Schott und Gen., Jena). Clear and almost colourless filtrates were obtained.

In Table 19 the results of these extraction experiments will be seen.

From the Table it is clearly seen that no complete extraction took place until a borate buffer was used. By using this buffer a marked optimum could be observed at pH 9.0. At this pH the extraction seems to be fairly complete, since the control preparation gives about the same quantities of reducing sugar in the enzyme experiment.

The reason for trying a borate buffer as an extraction fluid is not far to seek: the mucilage in which the bacterial cells are embedded, is soluble in alkaline solutions, and it might be supposed that a large quantity of the enzymes would be adsorbed by the mucilage or at least prevented from free diffusion into the solution. By treatment with an alkaline solution the mucilage would be partly dissolved. On the other hand, too strong alkaline media must be dangerous to the enzymes and decrease their activity.

Sodium hydroxide and ammonia have been occasionally tried instead of borate buffer for extraction of the bacteria preparations, likewise with a good result. Ammonia has the advantage of being easily evaporated after the extraction. Further experiments are being made with this substance.

Table 10. Extraction of Dry Bacteria (C-Preparation) of *Cytophaga globulosa* by means of Various Solutions.

Experiment	Extracting liquid	Time in days	Temp. °C	Mg of glucose formed by 1 ml extract (5 mg dry preparation) after	
				2 hours	6 hours
1.	Water	1	0	5.9	9.0
	» + toluene	1	0	5.3	7.8
	» »	1	20	6.1	8.7
2.	Water	4	0	6.0	8.9
	» + toluene	4	0	7.5	9.0
	» »	4	20	7.7	10.2
3.	Phosphate buffer pH 7.0	5	0	8.4	7.3
	» + toluene	5	0	3.9	7.4
	» »	5	20	7.6	11.1
4.	Borate buffer pH 8	1	20	5.5	8.4
	» pH 9	1	20	12.2	18.8
	» pH 10	1	20	8.3	12.1
(all with toluene)					
Control preparation 5 mg				11.3	17.1
» » 10 mg				19.0	25.6

A more accurate pH experiment was made in order to establish the optimum value for the extraction with borate. The range from pH 8 to 10 with intervals of 0.5 pH was tested. Buffer solutions having the exact values of 8.0, 8.5, 9.0, 9.5 and 10.0 were made up.¹ Through the addition of bacteria preparation the values were somewhat lowered, and after the extraction still lower values were registered:

pH of the buffer	pH after addition of bacteria	pH after extraction
8.0	7.5	7.3
8.5	8.2	8.0
9.0	8.8	8.4
9.5	9.4	9.4
10.0	9.8	8.9

The preparations were extracted in the respective solutions for 20 hours at room temperature (20° C), centrifuged and filtered as described above.

¹ The pH values determined electrometrically by means of a Beckman potentiometer with glass electrode; determined with Lypheane papers the same figures were obtained.

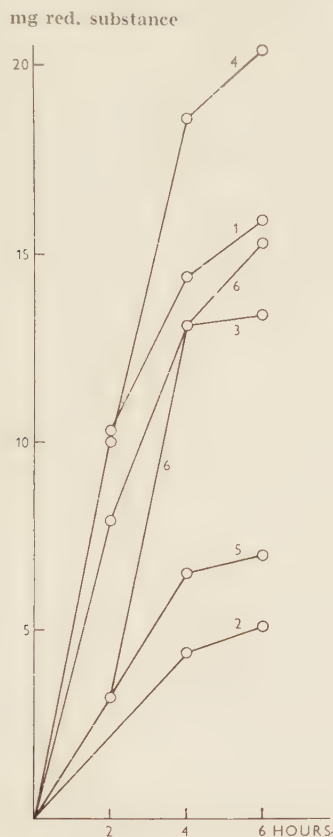


Fig. 23. Hydrolysis of lichenin by borate buffer extracts of dry bacteria preparations. The preparations are 1) unextracted (5 mg), 2) extracted at pH 7.5, 3) at 8.2, 4) at 8.8, 5) at 9.4, 6) at 9.8. C-preparation.

and finally neutralized. 1 ml amounts corresponding to 5 mg dry preparation were then tested in a usual enzyme experiment. The values obtained are seen from the accompanying curves (Fig. 23).

The optimum earlier found is confirmed by this experiment. A most complete extraction appears to take place at pH 8.8 (8.4 after the extraction), whereas pH 9.4 apparently causes a partial destruction of the enzyme system. Curve 6 may perhaps be less reliable, since the pH value during the extraction changed considerably. At pH 8.2 (8.0 after extraction) a fairly good extracting capacity of the buffer solution can also be observed. During the extraction with borate it seems important to maintain a low temperature, since in general more active solutions were obtained under such circumstances.

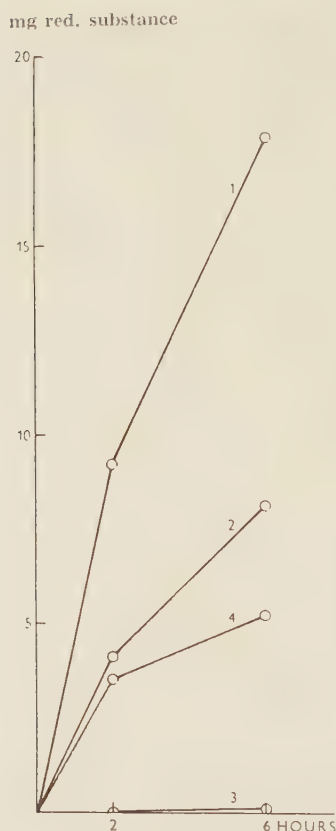


Figure 24. Hydrolysis of lichenin by dialyzed borate buffer extracts of dry bacteria preparations. 1) Undialyzed solution, 1 ml, 2) dialyzed solution, 1 ml, 3) outer solution, 2 ml, 4) inner solution, 1 ml + outer solution, 2 ml.

After extraction with borate the solution is turbid, as mentioned above. A clarification of the solution is facilitated by precipitation with $\text{CaCl}_2 + \text{K}_2\text{HPO}_4$, a method used by DUBOS when preparing cell-free extracts of *Pneumococcus* (cf. BAMANN and MYRBÄCK 1941 p. 1211). The precipitate formed adsorbs the colloidal substances of the suspension and the precipitate can be easily centrifuged off.

To remove the salts from such extracts dialysis was tried. Several experiments were carried out using collodion sacs for this purpose, but invariably the dialysis experiments have resulted in a considerable decrease in the activity of the solutions. Only a very slight diffusion of the enzymes concerned through the membrane seems to take place, however. The conditions can be seen from Fig. 24, which gives the results from a dialysis

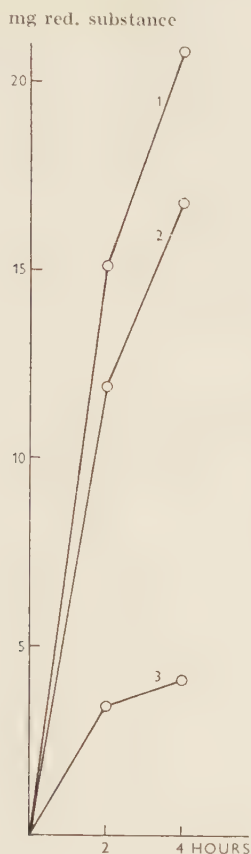


Fig. 25. Hydrolysis of lichenin by an enzyme solution. 1) Non-dialyzed, 2) dialyzed for 1.5 hours and 3) for 20 hours.

experiment with 10 ml borate extract dialyzed against 20 ml distilled water for 20 hours at $+3^{\circ}\text{C}$.

As is evident from the Figure, a considerable part of the enzyme activity has been lost through the dialysis. The lost activity cannot be recovered from the outer solution. In a separate experiment it was found that the activity of the dialyzed solutions was not increased by addition of Na, Mg or Ca (given as chlorides). The possibility remains that part of the lichenase is adsorbed at the surface of the collodion membrane. As collodion is a cellulose derivative, the cellulose-splitting enzymes may have a certain affinity to this substance.¹

¹ Collodion is not utilized as a substrate by *Cytophaga*.

The shape of curve 4 may indicate that some inhibitory substances present in the enzyme solution diffused through the membrane.

The inactivation at dialysis by means of collodion sacs appears to proceed very rapidly. In an experiment samples were taken out from the dialyzed liquid after 1.5 hours and after 20 hours. The dialysis was made against running tap water ($+ 10^{\circ}\text{C}$). The result obtained will be seen in the accompanying curves (Fig. 25).

Dialysis by means of an animal membrane (calf gut) has also been tried but without success. A decrease in activity of the enzymes concerned has been observed also in this case.

D. The Hydrolysis of Cellophane.

In studies of the enzymatic decomposition of true cellulose, mainly regenerated cellulose has been used. Cellulose precipitated from a solution in highly concentrated HCl was employed by GRASSMANN and associates (1933 a). Cellophane produced from viscose or cuprammonium solution has also been used by several authors. Because there are uniform qualities of cellophane commercially available, this substrate offers certain advantages, and the present writer has used cellophane in most experiments with *Cytophaga* cellulase.

Cotton cellulose is far more resistant to enzyme action than regenerated cellulose, as was clearly established already by the investigations of KARRER, SCHUBERT and WEHRLI (1925) into the *Helix* cellulase. In my preliminary trials with *Cytophaga* cellulase I have not obtained measurable amounts of reducing sugar by enzyme action upon cotton cellulose.

Independently of the question whether the cellulase and lichenase are specifically distinct from each other or not in the bacteria here concerned, it appeared as though methods which increased the lichenase activity also raised the cellulase activity in the preparations. Since working with lichenin as a substrate was much simpler and gave much higher hydrolysis figures in shorter times, most tests in the preliminary experiments for increasing the activity of the preparations were carried out using lichenin as a substrate. Occasionally experiments with cellophane were also made. The first preparations exhibited only a weak action upon cellophane, but parallelly with an increased action on lichenin an increased action on cellophane was also registered.

Some difficulties were met with in the first experiments with cellophane, various kinds tested being completely resistant to the enzyme action and even to the attack of living bacteria. An appreciable difference seemed to exist between different kinds of cellophane, however. KARRER, SCHUBERT

and WEHRLI (1925) have earlier pointed out the great resistance of certain forms of cellophane.

Cellophane may be of a somewhat varying constitution in respect to molecular weight, crystalline structure, etc. STAUDINGER and FEUERSTEIN (1936) found a D. P. of 270—280 in cellophane "Kalle". The conditions at the precipitation may influence the structure. Compare the reports on cotton cellulose and lichenin on pp. 74 and 84.

As a preliminary experiment, therefore, a number of different cellophane samples were tested as substrate for *Cytophaga globulosa* in a usual growth experiment, since the suitability under these circumstances seemed to be a necessary condition for successful enzyme experiments. Cellophane strips of different makes were consequently tested in tubes with Waksman's medium and the cellulose decomposition estimated.

The kinds of cellophane used were mainly of Swedish make. The descriptive figures in the Table below indicate the weight in g per m². Some, i. e., A 30, D 30, were of German make. All Swedish cellophanes are produced by means of the viscose process.

The results obtained are seen in Table 20.

It is evident from the Table that the resistance to bacterial attack of the various kinds of cellophane is very different. This may explain the difficulties of STANIER (1942 b, p. 170) in cultivating *Cytophaga* on cellophane. The samples are arranged after increasing thickness, and the thickness of the different kinds consequently cannot be of importance. It is

Table 20. Decomposition of Cellophane Strips by *Cytophaga globulosa*.

3 Parallel Tubes. Temperature 30 °C.

Kind of cellulose	Decomposition after days				
	2	3	5	7	9
Filter paper 0 B	+	++	+++		
Cellophane A 30 (37 g/m ²)	—	+	+	++	+++
» D 30 (23 g/m ²)	—	—	—	—	—
» 31	—	—	—	—	+
» 36	—	+	+	++	+++
» 40	—	—	+	+	++
» 53	—	—	—	+	++
» 54	—	—	—	—	—
» 59	—	—	+	++	++
» 61	—	+	++	+++	

— no growth

+++ maximal growth

remarkable that filter paper, i. e. native cellulose, is more easily attacked than any of the cellophanes. It is possibly only due to physical conditions, the filter papers being more saturated with water, etc., or is perhaps due to some growth factors present in the filter papers, while the cellophane strips are purer in this respect.

A second experiment was set up, using the most suitable of the above kinds of cellophane and in addition some kinds of cellophane tubing cut in strips. The result of this experiment is seen from Table 21.

Table 21. Decomposition of Cellophane Strips by *Cytophaga globulosa*.

3 Parallel Tubes. Temperature 30° C.

Kind of cellulose	Decomposition after days				
	2	3	4	7	11
Filter paper 0 B	—	—	—	—	—
Cellophane A 30	—	—	+	+++	—
» 36	—	—	+	+++	—
» 59	—	—	+	+	+++
» 61	—	—	+	++	+++
» T (94 g/m ²)	—	+	+	++	+++
» S (107 g m ²)	—	—	—	—	—

— no growth

+++ maximal growth

This experiment gave results agreeing with those in the preceding one. The comparatively low resistance characterizing tubing T explains why this tubing is not suitable in dialysis work with bacteria preparations, as was frequently observed. Even the more resistant tubing S, however, is not perfectly reliable in this respect even at a temperature of 0° C.

As a consequence of the experiments carried out, cellophane 61 was chosen for the subsequent work. This make was obtained from A/B Nordisk Silkecellulosa, Norrköping.

In an enzyme experiment the best cellophane qualities, as found in the above growth experiment, were compared. The results will be seen from the curves given in Fig. 26.

From the curves a certain variation in susceptibility of the different cellophane qualities is evident. Thus cellophane 36 was not attacked at all (not plotted in the Figure), whereas cellophane 61 shows the most rapid hydrolysis.

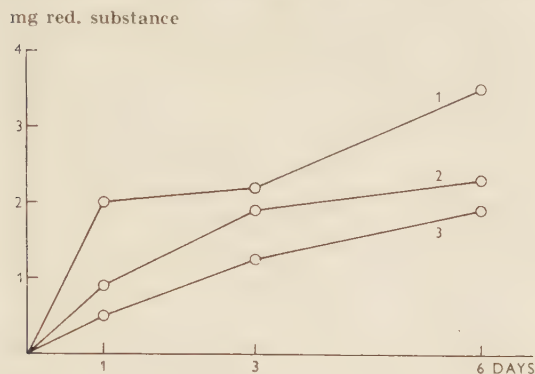


Figure 26. Hydrolysis of various kinds of cellophane. 1) Cellophane 61, 2) cellophane A 30, 3) cellophane tubing T. 100 mg cellophane in each flask. 20 mg C-preparation. Temperature 30° C.

One important point in using a solid substrate such as cellophane is the quantity exposed to enzyme action. For the *Helix* cellulase and *Aspergillus* cellulase this was clearly established by KARRER and associates (1923) and GRASSMANN and associates (1933 a). The hydrolysis is not relatively, but absolutely, more rapid at a higher concentration of cellophane, a greater surface being exposed to the enzyme action.

Also regarding the *Cytophaga* cellulase this fact is obvious.

An experiment carried out with increasing amounts of cellophane gave a result which will be seen in the accompanying figure (Fig. 27).

From Fig. 27 the conclusion can be drawn that a higher rate of hydrolysis is obtained with a greater quantity of substrate.

A pH experiment was carried out using cellophane as a substrate. The various pH values were adjusted as in the lichenin experiment with mixtures of primary and secondary phosphate according to SÖRENSEN. The following values were obtained:

Initial pH	pH after 3 days
5.4	5.3
6.0	5.9
6.9	6.7
7.3	7.2
7.4	7.4

The result is seen from Fig. 28.

In this experiment the optimum apparently lies at about the same value as in the case of lichenin. A peak is observed at pH 7, although the differences are not so great. At pH 5.4 the velocity of the hydrolysis is considerably less. The cellulase from *luizym* has its optimal action at 4.8,

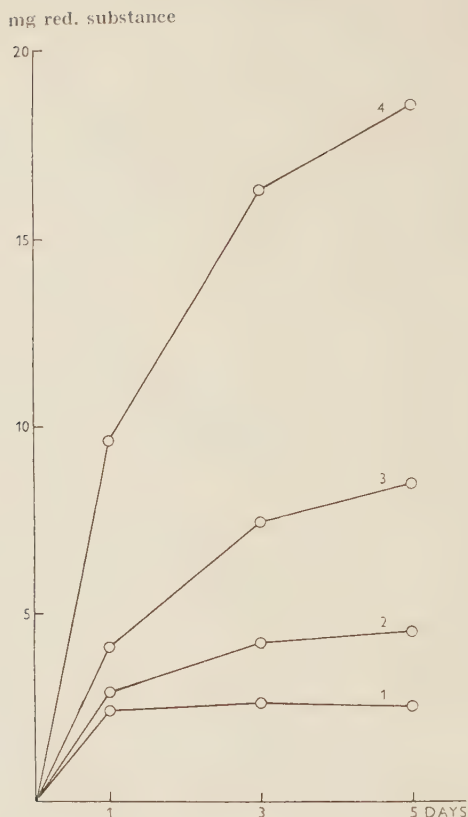


Fig. 27. Hydrolysis of increasing amounts of cellophane. 1) 50 mg of cellophane in each flask, 2) 100 mg, 3) 200 mg, 4) 500 mg. 20 mg H-preparation. 2 parallel flasks. Temperature 30° C.

and that from *Helix* juice at about 5.3 (FREUDENBERG and PLOETZ 1939). GRASSMANN and collaborators (1933 a) found a somewhat lower optimum for cellulase, using a suspension of precipitated cellulose, whilst FREUDENBERG and PLOETZ used cuprophane folia. The preparations may have been somewhat different in respect to purity. The only bacterial cellulase so far investigated in this respect, namely that of *Cellulobacillus myxogenes* (SIMOLA 1931 b), has also an optimum different from that of the *Cytophaga* enzyme here concerned. SIMOLA found that the most rapid hydrolysis took place within the range 5.0—6.0, which is thus lower than for *Cytophaga globulosa*.

An experiment was carried out with the addition of increasing amounts of glucose in order to make clear whether the cellulase was

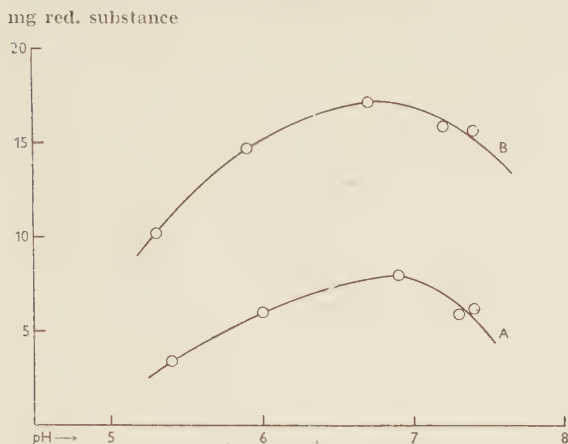


Figure 28. Hydrolysis of cellophane (500 mg) at various pH values. A 1 day, B 7 days, 20 mg C-preparation, 2 parallel flasks. Temperature 30° C.

inhibited by glucose (cf. the earlier experiment with lichenin, p. 96). The result of the experiment will be seen from Fig. 29.

As seen from the Fig., the results are similar to those obtained with lichenin as substrate.



Fig. 29. Hydrolysis of cellophane (500 mg) on addition of increasing amounts of glucose. 1) No addition, 2) 0.05 % glucose, 3) 0.1 %, 4) 0.25 %, 5) 0.5 %, 20 mg H-preparation. Temperature 36° C.

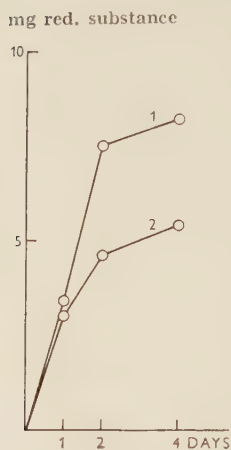


Figure 30. Hydrolysis of cellophane with (1) and without (2) addition of MgCl_2 . 100 mg cellophane in each flask. 20 mg C-preparation. Temperature 30°C .

The addition of Mg-ions seems to have a favourable influence in the hydrolysis of cellophane also, as is seen from Fig. 30. It may be noted that Mg was added also in the experiments related above (Figs. 27—29).

A certain stimulation through Mg-ions has apparently taken place.

The cellulase is extractible by treatment of the dry bacteria preparations with a borate buffer or ammonia at pH 9, as was found regarding the lichenase (see p. 105 above). These extracts have a somewhat lower cellulase activity than have the untreated preparations. Compare Fig. 2 in an earlier report (FÄHRÆUS 1946). The enzyme solution obtained during the freezing of the precipitated bacteria, as reported on p. 103 above, has a good activity upon cellophane. Such a solution has been used in the experiments to identify the reducing substance formed from cellophane (p. 72).

It was observed that the cellophane strips used in the enzyme experiments underwent a considerable reduction in tensile strength. This offers a method for the study of the course of enzyme action, but no accurate experiments have so far been carried out along these lines.

E. The Hydrolysis of Cellobiose and Other Carbohydrates.

Judging from the results obtained with living *Cytophaga* cells, no other substances than cellulose (incl. lichenin), cellobiose, glucose and mannose were suitable as sources of carbon (pp. 38, 47). The enzymatic methods later employed offered a simple means of testing the suitability of different polysaccharides and oligosaccharides.

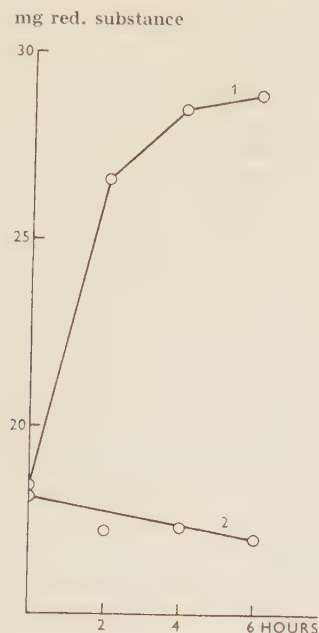


Fig. 31. Hydrolysis of 1) cellobiose and 2) maltose by 0.2 ml cell-free enzyme solution, containing about 2 mg dry substance. 2 parallel flasks. Temperature 36°C .

That cellobiose was readily hydrolyzed by the *Cytophaga* preparations was already probable from the experiments reported on p. 72. The following substances¹ were also tried as substrates for *Cytophaga globulosa* in enzyme experiments: soluble starch, dextran, yeast mannan, salicin, raffinose, trehalose, maltose, lactose and sucrose. All these compounds were, however, found unsuitable for the *Cytophaga* preparations. The substances named were tested in 0.02 M solutions (as glucose). The result of such an experiment with maltose and cellobiose will be seen from Fig. 31.

The curves show that, while the cellobiose was readily hydrolyzed, the maltose remained unattacked, the reducing value being fairly constant during the course of the experiment.

¹ The salicin and yeast mannan were kindly provided by Professor K. MYRBÄCK, University of Stockholm. The salicin had a sp. rot. of $[\alpha]_{\text{D}}^{20^{\circ}} = -62.7^{\circ}$. The mannan had been prepared from baker's yeast (see LINDSTEDT, Ark. Kemi 20, No. 13, 1945) and had a sp. rot. of $[\alpha]_{\text{D}}^{20^{\circ}} = +88^{\circ}$. The dextran was obtained through the courtesy of Docent A. GRÖNWALL, Akademiska Sjukhuset, Uppsala. This polysaccharide had been prepared from cultures of *Leuconostoc mesenteroides*. It had been subjected to partial hydrolysis with acid.

The other substances employed were: soluble starch, "analar", raffinose "c. p. Pfanstiehl", trehalose "c. p. Pfanstiehl", maltose "reinst Schering", lactose "DAB 6 Schering", sucrose "reinst für Injektionen, Merck".

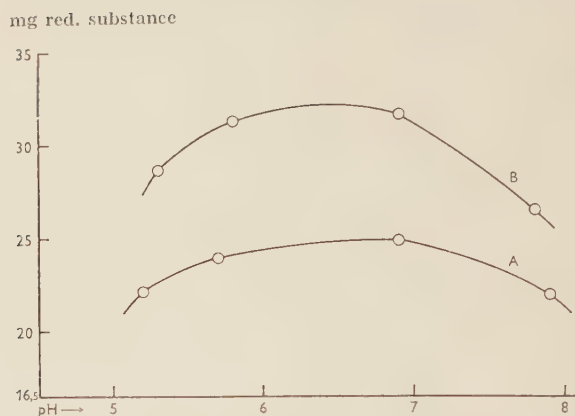


Fig. 32. Hydrolysis of cellobiose at various pH values. A 1 hour, B 4 hours. 5 mg H-preparation. Temperature 36°C .

It may be noted that similar results with the disaccharides have been obtained both when using H-preparations and when using C-preparations. The unsuitability of maltose, etc., is consequently not connected with an inactivation of the enzymes concerned by the low pH value at the preparation.

It is of interest to observe that the β -glycoside salicin is not hydrolyzed by the *Cytophaga* preparations. Although the *Cytophaga* cellobiase is a β -glucosidase, it is thus distinct from the salicinase from different sources (cf. p. 78 above).

A pH experiment was carried out using cellobiose as a substrate. The arrangements were the same as in the previously reported experiment with lichenin (p. 87). The results will be seen from Fig. 32.

The optimum for the cellobiase activity seems to lie within the same range as that for the lichenase and cellulase. The curves appear to be a little more flat than in the case of lichenin and cellophane, however.

Summary.

The purpose of this work is to throw light upon the cellulose decomposition by *Cytophaga*, a bacterial group that has aroused much interest and provoked much controversy ever since the discovery of the first representative of this genus by HUTCHINSON and CLAYTON in 1919. Many investigators have been engaged in researches into this subject. The main course of the cellulose decomposition brought about by these bacteria has, however, remained rather obscure up to recent years.

The contents of this paper may be summarized as follows.

1. The taxonomy and morphology of *Cytophaga* are briefly surveyed, in so far as they seem important for an understanding of the physiological problems under consideration. The author has not made investigations of his own into these matters.
2. The bacterial strains used are *Cytophaga 3*, isolated from garden soil in 1937, and *Cytophaga globulosa*, described by STAPP and BORTELS (1934) and isolated from beech-wood litter. The morphology of these strains largely conforms with that of earlier described microcyst-forming *Cytophaga* strains. The procedure adopted in isolating *Cytophaga 3* is related.
3. Methods of cultivating these organisms are discussed; the procedure followed in the quantitative experiments is given. The method for determining residual cellulose in *Cytophaga* cultures is described.
4. The nutrient requirements and pH conditions are discussed. Ca and Mn appear to stimulate the growth of *Cytophaga 3*, but no accurate quantitative experiments were carried out. A pH experiment with *Cytophaga 3* indicated that the optimum lies about the neutral point. The upper and lower limits for growth seem to lie at about 5.5 and 8.5 respectively (Tab. 1, Fig. 1).
5. It was found that *Cytophaga 3* formed considerable quantities of thiamin, as demonstrated with the *Phycomyces* method (Fig. 2). A qualitative experiment seemed to show that thiamin stimulates the initial growth of *Cytophaga 3*.

6. The suitability of various sources of cellulose is discussed. It was found that certain kinds of cellulose contained growth-inhibiting substances, which fact caused some difficulties in cultivating these bacteria, especially on agar plates with filter paper. In most growth experiments cellulose-wadding or cotton-wool were used as substrates.
7. The influence of soluble carbohydrates on the growth and cellulose decomposition by *Cytophaga* has been studied. The statements in the literature are briefly related and some weak points indicated. Qualitative experiments with various sugars carried out by the author gave results agreeing, on the whole, with those earlier obtained by others. In the case of glucose, however, a different result was registered, *Cytophaga* 3 being able to grow on glucose solutions of 0.25 and 0.5 % (Tab. 5). Cellobiose and mannose were also utilized.
8. Quantitative experiments were carried out with cellulose and glucose simultaneously present as substrates for *Cytophaga* 3. The results showed that glucose was preferentially used as a source of carbon and that the cellulose was not attacked until most of the glucose had been consumed (Tabs. 7, 9, Figs. 3, 4, 5, 7). If larger amounts of glucose were added to cultures with vigorous cellulose decomposition, this decomposition was broken off, and the glucose was utilized first. An inhibitory effect of higher glucose concentrations was noted in accordance with statements in the literature. Substantially the same results were obtained with *Cytophaga globulosa* (Tabs. 8, 9, Figs. 6, 8).
9. The inhibitory action of higher concentrations of glucose is discussed. The result of STANIER (1942 b) to the effect that the inhibition is due to toxic substances formed in the glucose solutions on heat sterilization, has not been confirmed by my experiments. A definite solution to this problem has not yet been found.
10. The course of cellulose decomposition by *Cytophaga* has been subjected to closer investigation. The oxydative theory is rejected, and a hydrolytic course of decomposition is assumed on the following grounds. The *Cytophaga* mucilage, regarded as a kind of oxycellulose by several authors, is most probably a purely synthetic product of the bacterial cell, as it is formed in both glucose and cellulose media (Tab. 10, Fig. 9). The hydrolysis of cellulose, which could not be demonstrated in living cultures of *Cytophaga*, was established in enzyme experiments with easily split cellulose sources, such as regenerated cellulose and lichenin (Figs. 10 et seq.). The glucose arising, regarded unsuitable as a source of carbon by many investigators, is readily utilized by *Cytophaga*, as mentioned above. Consequently there seems to be no

obstacle to the assumption of a hydrolytic course of decomposition. Compare also the summary on p. 76.

11. Bacteria and enzyme preparations of *Cytophaga* were produced by different methods. With *Cytophaga globulosa* the procedure was most simple, and preparations of this strain have been used for a number of enzyme experiments. As substrates lichenin and cellophane were mainly used. Details regarding the bacteria preparations, substrates, and arrangement of the experiments are given.
12. The influence of temperature, pH, phosphate concentration, various salts, etc., on the lichenase activity was studied (Figs. 11—20). The pH optimum was found to lie at the neutral point like the pH optimum of living bacteria. An interesting result was that Mg ions activated the preparations appreciably. Glucose retarded but did not prevent the hydrolysis process (higher concentrations than 0.5 % were not tried).
13. Attempts were made to improve the activity of the bacteria preparations. Rapid work at low temperature and alternate freezing and thawing of the bacteria seems to raise the activity.
14. An extraction of the cellulose-decomposing enzyme system from the dried bacteria was tried. Fairly good results were obtained by using a borate buffer at pH 8—9 (Fig. 23). By subsequent filtration cell-free solutions containing the enzymes concerned could be obtained. Dialysis of such solutions resulted in a considerable inactivation of the enzyme system (Figs. 24, 25).
15. Experiments with cellophane as substrate gave results similar to those with lichenin, although the rate of hydrolysis was very much lower. Various kinds of cellophane showed very different resistance to enzyme action. A great concentration of cellophane increased the yield of reducing substance in the enzyme experiments. Mg ions appear to stimulate the hydrolysis of cellophane, too. (Figs. 26—30.)
16. Cellobiose is readily hydrolyzed by the *Cytophaga* preparations. A pH experiment with cellobiose as substrate gave about the same optimum as that earlier obtained for lichenin and cellophane (Fig. 32).
17. The following substances were tried as substrates, but with an entirely negative result: starch, dextran, yeast mannan, salicin, raffinose, maltose, trehalose, lactose, sucrose.

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PLATES I—II.

The preparations 1, 2, 4, 5, and 6 were fixed by drying on the slide and stained with carbol-fuchsin. They were immersed in oil and photographed with light-field illumination. Objective: Zeiss apochromate num. ap. 1.30 ($\times 90$).

The preparations 3 and 7 were fixed by means of osmic acid vapours and immersed in water under a cover slip. They were photographed with dark-field illumination. Objective: Zeiss apochromate num. ap. 0.7 ($\times 60$). Condenser: Zeiss "Präparier-Wechsel-Kondensor" num. ap. 0.7.

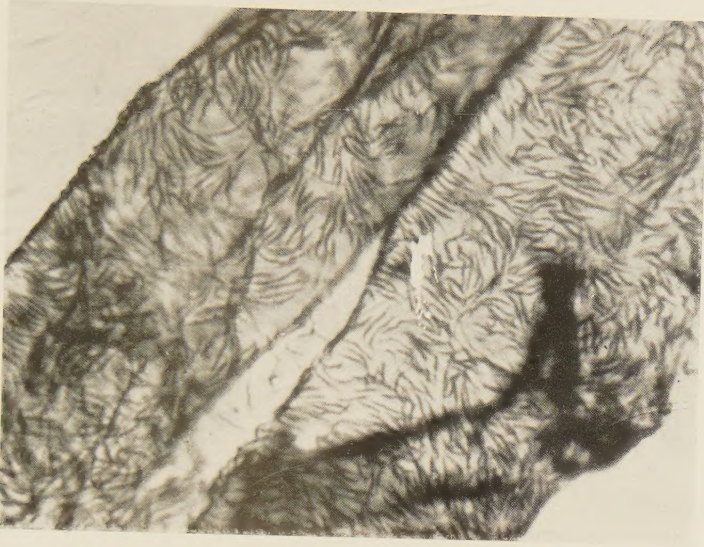


Fig. 1



Fig. 2

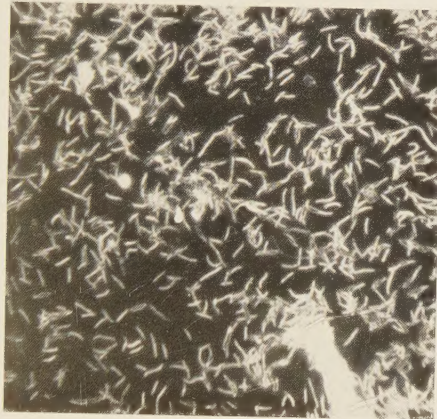


Fig. 3

Fig. 1. *Cytophaga* 3. $\times 1400$. Cellulose fibres thickly set with the thread form.

Fig. 2. *Cytophaga* 3. $\times 1400$. Fragment of a fibre occupied by cells.

Fig. 3. *Cytophaga globulosa*. $\times 600$. Thread form. Observe the rounded ends of the cells. Compare STANIER 1942 b p. 149.

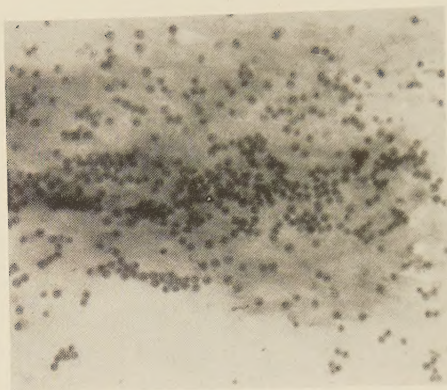


Fig. 4

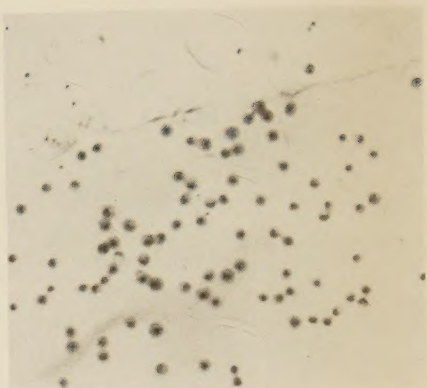


Fig. 5

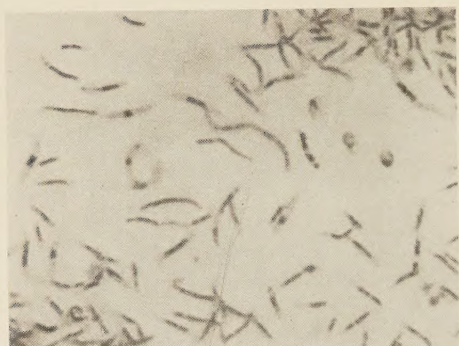


Fig. 6

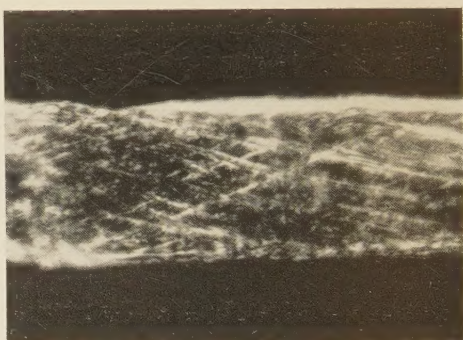


Fig. 7

Fig. 4. *Cytophaga 3*. $\times 900$. Microcysts embedded in mucilage.

Fig. 5. *Cytophaga 3*. $\times 1400$. Microcysts showing the distribution of the chromatin.

Fig. 6. *Cytophaga 3*. $\times 1400$. Cells of varying shape resulting from the addition of 0.5 % glucose.

Fig. 7. Partly disintegrated cotton fibre, showing the spiral structure. $\times 600$.

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